

# **The peptidyl-prolyl isomerase Pin1 is required for maintenance of the spindle assembly checkpoint**

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**Andreas Brown**  
aus Starnberg

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Amtierende Dekanin:

Prof. Dr. Beate Lohnert

Prüfungsausschuss:

Prof. Dr. Olaf Stemmann (Erstgutachter)

Prof. Dr. Franz-Xaver Schmid (Zweitgutachter)

Prof. Dr. Klaus Ersfeld (Vorsitzender der Prüfungskommission)

Prof. Dr. Matthias Weiss

Prof. Dr. Birgitta Wöhr





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## SUMMARY

Chromosomes are replicated during S-phase and segregated during M-phase of the eukaryotic cell cycle. The two sister chromatids of each duplicated chromosome are topologically entrapped and, thus, paired by the ring-shaped protein complex cohesin. They are separated in anaphase of mitosis when cohesin is endoproteolytically cleaved by separase. Activation of this giant protease requires the degradation of its two inhibitors, securin and cyclin B1, which is mediated by the anaphase promoting complex or cyclosome (APC/C), a multisubunit ubiquitin ligase, in conjunction with its essential co-activator Cdc20.

The spindle assembly checkpoint (SAC) is a surveillance mechanism that monitors the chromosomes' interactions with the microtubules of the mitotic spindle apparatus. In response to even one erroneous attachment the affected kinetochore emits a "wait anaphase" signal, which is amplified and culminates in the quantitative sequestration of Cdc20 by the SAC components Mad2 and BubR1. The consequent inactivation of the APC/C causes a metaphase arrest and gives the cell time to correct the error. Given its great importance for chromosome segregation fidelity, it comes at no surprise that loss of the SAC causes cell death while its curtailing is associated with tumour formation.

Pin1 is a peptidyl-prolyl-isomerase with strong preference for phosphorylated Ser-Pro or Thr-Pro motives within its protein substrates. In the present thesis, evidence for the involvement of Pin1 in the maintenance of a robust SAC response is presented.

Antibodies against Pin1 were raised and used to establish the effective immunodepletion of Pin1 from extracts of *Xenopus laevis* eggs. While the SAC could readily be activated in mock-treated samples of this cell free system, securin was degraded despite the presence of unattached kinetochores when Pin1 had previously been removed. Proving the specificity of this effect, a SAC mediated arrest could be rescued by adding back recombinant Pin1 to depleted extracts. Similarly, addition of dominant negative but not of wild-type Pin1 to SAC-arrested extracts resulted in a checkpoint override.

Chemical inhibition of human Pin1 with two different molecules in two different cancer cells lines invariably forced the cells to exit mitosis in the absence of spindles. This resulted in the premature disappearance of securin, cyclin B1 and a mitosis-specific phos-

phorylation on Ser10 of histone H3. Thus, Pin1's role as a checkpoint component is conserved in mammals.

In search for the relevant target, Cdc20 was identified as a novel interaction partner of vertebrate Pin1. This association requires phosphorylation of Cdc20 on Ser-Pro/Thr-Pro sites and occurs only during mitosis. Importantly, the Pin1-Cdc20 interaction is direct and not bridged via another checkpoint component or a subunit of the core APC/C. The experimental data suggest that Pin1-dependent isomerization of Cdc20 might bias it to preferentially associate with Mad2 and BubR1 instead of APC/C.

Taken together, these findings contribute to a better understanding of the molecular mechanisms involved in SAC signalling and unravel a previously unappreciated role of Pin1 for genome integrity.

## ZUSAMMENFASSUNG

Chromosomen werden während der S-Phase des eukaryotischen Zellzyklus repliziert und während der M-Phase voneinander getrennt. Die beiden Schwesterchromatiden eines jeden duplizierten Chromosoms sind räumlich verbunden und mit Kohäsin, einem ringförmigen Protein-Komplex, gepaart. Sie werden in der Anaphase der Mitose voneinander getrennt, nachdem Kohäsin endoproteolytisch durch Separase gespalten wurde. Die Aktivierung dieser großen Protease erfordert den Abbau seiner beiden Inhibitoren, Securin und Cyclin B1, was vom Anaphase Promoting-Komplex oder Zyklusom (APC/C), einer aus mehreren Untereinheiten bestehenden Ubiquitin-Ligase in Verbindung mit seinem wichtigen Co-Aktivator Cdc20, bewerkstelligt wird.

Der „Spindle Assembly Checkpoint“ (SAC) ist ein Kontrollmechanismus, der die Anheftungen der Chromosomen mit den Mikrotubuli des mitotischen Spindelapparats überwacht. Als Antwort auf eine einzelne fehlerhafte Befestigung sendet das betroffene Kinetochor ein „*Wait anaphase*“-Signal aus, welches vielfach verstärkt wird und in der quantitativen Sequestrierung von Cdc20 durch die SAC-Komponenten Mad2 und BubR1 resultiert. Die konsequente Inaktivierung des APC/C verursacht einen Metaphase-Arrest und gibt der Zelle nun Zeit, den Fehler zu korrigieren. Angesichts seiner großen Bedeutung für die Chromosomensegregation, ist es nicht verwunderlich, dass der Verlust der SAC-Funktion den Zelltod verursacht, während seine Beeinträchtigung mit der Entstehung von Tumoren einhergeht.

Pin1 ist eine Peptidyl-Prolyl-Isomerase mit starker Präferenz für phosphorylierte Ser-Pro- oder Thr-Pro-Motive innerhalb seiner Proteinsubstrate. In der hier vorliegenden Arbeit werden Hinweise für die Beteiligung von Pin1 im Aufrechterhalten einer robusten SAC-Antwort präsentiert.

Es wurden Antikörper gegen Pin1 hergestellt und dazu genutzt, die effektive Immunodepletion von Pin1 aus Extrakten von *Xenopus laevis* Eiern zu etablieren. Während der SAC in kontroll-behandelten Proben dieses zellfreien Systems aktiviert werden konnte, wurde Securin trotz der Anwesenheit von freien Kinetochoren abgebaut, wenn Pin1 zuvor entfernt worden war. Ein SAC-vermittelter Arrest konnte durch Zugabe von rekombinantem Pin1 zu depletiertem Extrakt gerettet werden, was die Spezifität dieser Wir-

kung untermauert. Ebenso löste die Zugabe von dominant negativen, nicht aber von Wildtyp-Pin1 zu SAC-etablierten Extrakten, eine Deaktivierung des SACs aus.

Chemische Inhibierung des humanen Pin1 mit zwei verschiedenen Molekülen brachte zwei verschiedene Krebszellenlinien dazu, Mitose in Abwesenheit von Spindeln zu verlassen. Dies resultierte im Abbau von Securin, Cyclin B1 und Mitose-spezifischer Phosphorylierung des Serinrestes 10 von Histon H3. Somit ist die Rolle von Pin1 als SAC-Komponente bei Säugetieren konserviert.

Auf der Suche nach dem relevanten Ziel wurde Cdc20 als neuer Interaktionspartner von Wirbeltieren-Pin1 identifiziert. Diese Interaktion erfordert die Phosphorylierung der SP/TP-Aminosäurereste von Cdc20 und tritt nur während der Mitose auf. Wichtig ist, dass die Interaktion von Pin1 mit Cdc20 direkt ist und nicht durch eine andere Komponente des SAC oder einer Untereinheit des APC/C vermittelt ist. Die experimentellen Daten deuten darauf hin, dass Pin1-abhängige Isomerisierung von Cdc20 zu einer bevorzugten Assoziierung mit Mad2 und BubR1 anstelle des APC/C führt.

Zusammengenommen tragen diese Erkenntnisse zu einem besseren Verständnis der molekularen Mechanismen der SAC-Regulation bei und entschlüsseln eine bisher unbekannte Rolle von Pin1 für die genomische Integrität.

## **1. INTRODUCTION**

### **1.1. The eukaryotic cell cycle**

Continuous cell growth and cell division facilitate proliferation and reproduction and are fundamental features all organisms have in common. In order to enable accurate functionality and development of tissue, stable transfer of genetic information during cell division and to secure genomic integrity, the cell cycle is tightly regulated (Morgan, 2006).

The events of the cell cycle can be roughly divided into two stages, namely interphase and mitosis. Successful completion of the cell cycle requires cell growth and accurate duplication of the genetic material during interphase and its equal distribution between the two arising daughter cells during mitosis. Interphase itself is divided into the two “gap” phases G1 and G2, which enable the cell to grow and manufacture proteins required for the next phase, and furthermore S-phase, during which the actual process of DNA replication takes place (see Fig. 1).

The crucial events of the cell cycle are irreversible and uncorrected errors have severe consequence like cell death and aneuploidy, which can ultimately cause cancer. Therefore, timely and spatial control of cell cycle events is required and goes along with transcriptional regulation and reversible posttranslational modifications of distinct target proteins.

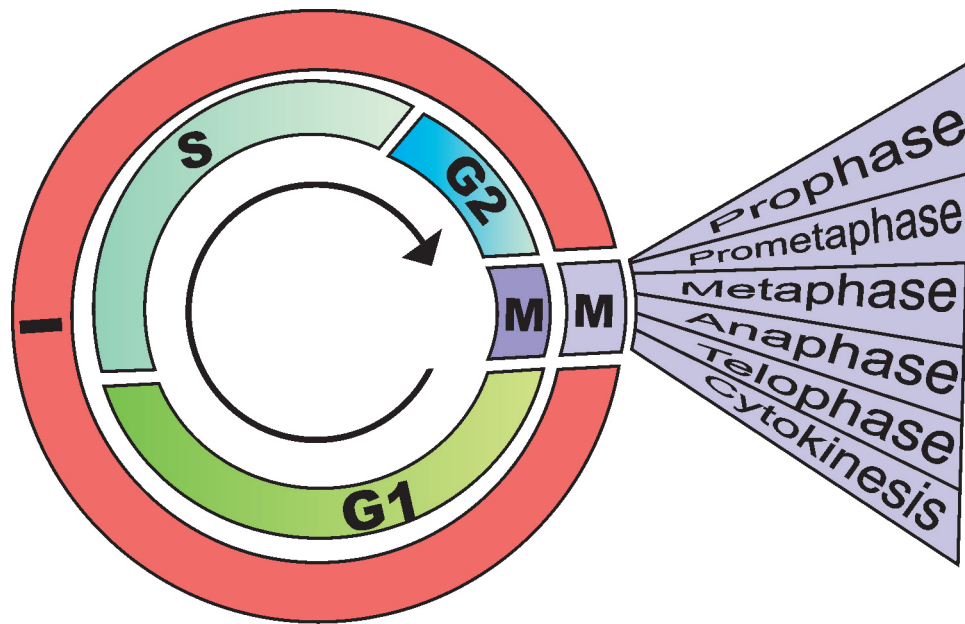
Key events of the cell cycle are triggered by a multitude of cyclin dependent kinases (Cdks) and their corresponding co-activators (named cyclins) whose expression levels depend on the current cell cycle stage. The most important kinase in mitosis is Cdk1 together with its cofactor cyclin B1. This key player phosphorylates a great range of proteins, among them being Cdc14, a phosphatase necessary for faithful exit from mitosis and several kinesin-like proteins necessary for proper spindle assembly (Millar et al., 1991; Ubersax et al., 2003).



## 1.2. Mitosis

After successful duplication of the genomic DNA in S phase, the eukaryotic cell prepares itself for the process of cell division, which includes mitosis and cytokinesis. During G2 phase, cells start to express mitosis-specific proteins, remove cell junctions to neighbouring cells and grow by adsorbing liquid. If any perceivable DNA damage persists, the DNA damage checkpoint is activated, arresting cells at the G2/M transition (Morgan, 2006).

Mitosis begins with prophase during which the chromatin begins to reorganize, forms higher order structures and condenses to densely packed chromosomes, each consisting of two identical and entrapped sister chromatids. Large proteinaceous structures, the kinetochores, start to assemble at the centromeric region of each sister chromatid. During prometaphase the nuclear envelope is dissolved, which is achieved by Cdk1-dependent phosphorylation of nuclear lamins leading to their rapid disassembly. Chromosomes now start to engage between the two poles of the cells, marked by the position of the centrosomes. The chromosomes bind to the mitotic spindle emanating from the microtubule organizing centres (MTOCs) via their kinetochores, assuring efficient spindle force between microtubules and DNA. Tight regulation of this process is imperative: Chromosomes must not divide until all chromosomes are appropriately attached to the mitotic spindle. A mechanism available for arresting the cell in the prometaphase state is the spindle assembly checkpoint (SAC). After a process of continuous oscillation of the chromosomes along the mitotic spindle, which can take several attempts until correct attachment and spindle force is achieved, the SAC is satisfied. In metaphase the chromosomes are arranged in an equatorial plane, the “metaphase plate” and sister chromatids are finally separated from each other. This segregation process marks the initiation of anaphase during which the now detached sister chromatids are pulled towards opposite poles. The mitotic spindle dissolves; lamins are dephosphorylated and start to assemble around the decondensing separated sister chromatids to form two nuclei in a process known as telophase. During the final stage, cytokinesis, the cytoplasm is divided to form two daughter cells with equal genomic and organelle content (Morgan, 2006; Fig. 1).



**Fig. 1: Overview of the eukaryotic cell cycle:** Roughly, the cell cycle is divided into interphase (I) and mitosis (M). Interphase can be further divided into G1, S and G2 phase, mitosis into prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. See text for details.

### 1.3. Cdk1, the key regulator of mitosis

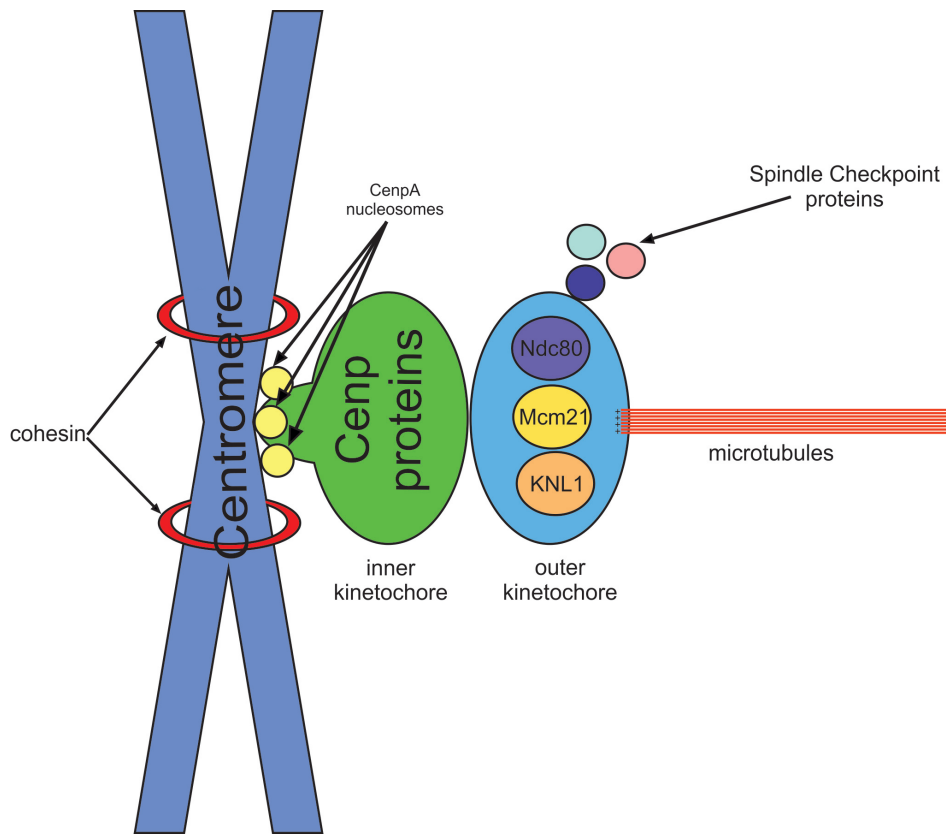
The most crucial kinase in mitosis is cyclin-dependent kinase 1 (Cdk1). This kinase phosphorylates serine or threonine residues, which are followed by proline and is activated upon mitotic entry. Cdk1 is a small, 34 kDa protein and contains a T-loop preventing enzymatic activity and substrate recognition in the absence of cyclins (Morgan, 2006). The fact that several hundred Cdk1 substrates have been identified, demonstrates the significance of this kinase for mitotic progression (Ubersax et al., 2003; Holt et al., 2009). Cdk1 phosphorylates a broad range of proteins involved in spindle assembly, checkpoint signalling, chromosome condensation, kinetochore assembly and nuclear envelope breakdown (Schmit and Ahmad, 2007).

Considering the major importance of Cdk1, the kinase has to be intrinsically regulated: Most important is its dependency on the presence of cyclin B1, which reaches its highest abundance upon mitotic entry and is destroyed at the metaphase-to-anaphase transition. Destruction is mediated by the E3 ubiquitin ligase APC/C, which recognizes a destruction box in cyclin B1 (Zur and Brandeis, 2002). Consequent cyclin B1 destruction in anaphase leads to complete and rapid Cdk1 deactivation. However, association with cyclin B1 is necessary but not sufficient for Cdk1 activity. Another important regulatory

function is executed by the kinases Wee1 and Myt1, which phosphorylate Cdk1 on residues T14 and Y15, thus promoting its inhibition (Parker and Piwnica-Worms, 1992). This mechanism is important to prevent premature mitotic entry during the G2/M transition. Upon mitotic entry, Cdc25 counteracts Wee1 activity and dephosphorylates Wee1-dependent Cdk1-residues, initiating Cdk1 activation (Timofeev et al., 2010).

#### **1.4. The kinetochore**

One of the major characteristics of mitosis is the presence of kinetochores, which orchestrate the distribution of sister chromatids to daughter cells. The kinetochore is a proteinaceous structure composed of several layers and situated at the centromeric region of mitotic chromosomes. The kinetochore serves as a centre for microtubule attachment, force establishment and spindle checkpoint signalling during mitosis (Wulf and Earnshaw, 2008). Roughly, the kinetochore can be divided into the inner and outer kinetochore, both of which are organized into further substructures. The inner kinetochore consists of a layer attached to the centromeric heterochromatin. Part of this layer are the Cenp proteins, among them being CenpA which associates with centromeric DNA and is a member of the histon H3 family (Musacchio and Salmon, 2007). The major component of the outer kinetochore is the outer plate essential for microtubule attachment. In budding yeast only one microtubule attaches to the outer plate, whereas in mammals it can be as many as 30 microtubules (Amor et al., 2004). Required for the microtubule-kinetochore attachment is the hetero-oligomeric Ndc80 complex, which directly binds to microtubules via its subcomponent Hec1 (Tooley et al., 2011). The KNL1 complex is also required for proper attachment of outer kinetochore components to microtubules and has a suggested role in spindle checkpoint silencing by recruiting phosphatase PP1 to the kinetochores (Cheeseman et al., 2008; Rosenberg et al., 2011). Another important component of the outer plate is the Mis12 complex, required for proper connection between the inner and outer kinetochore (Screpanti et al., 2011). Moreover, members of the SAC are also part of the outer kinetochore and enrich at the corona of the kinetochore in the absence of microtubules or in the presence of improperly attached microtubules. During the metaphase-to-anaphase transition, these proteins are rapidly removed (Howell et al., 2001).



**Fig. 2: Principal scheme of the kinetochore:** The kinetochore is a vast proteinaceous structure and can be roughly divided into two substructures: The inner kinetochore consists of Cenp proteins aligned to the centromeric regions of the mammalian chromosome. The outer kinetochore physically connects members of the spindle assembly checkpoint and the mitotic spindle apparatus. Some of its components are the KNL1, Mcm21 and Ndc80 complexes, which are essential for kinetochore-microtubule attachment and SAC signalling. See text for details. Please note that each mitotic chromosome possesses two kinetochores. For convenience, only one kinetochore is shown in this scheme.

### 1.5. Spindle assembly control

Considering the fact that uncorrected errors during the process of chromosome segregation are detrimental to cells and most cancers show chromosome aberrations, mitosis and especially the prometaphase-to-anaphase transition have to be solidly regulated.

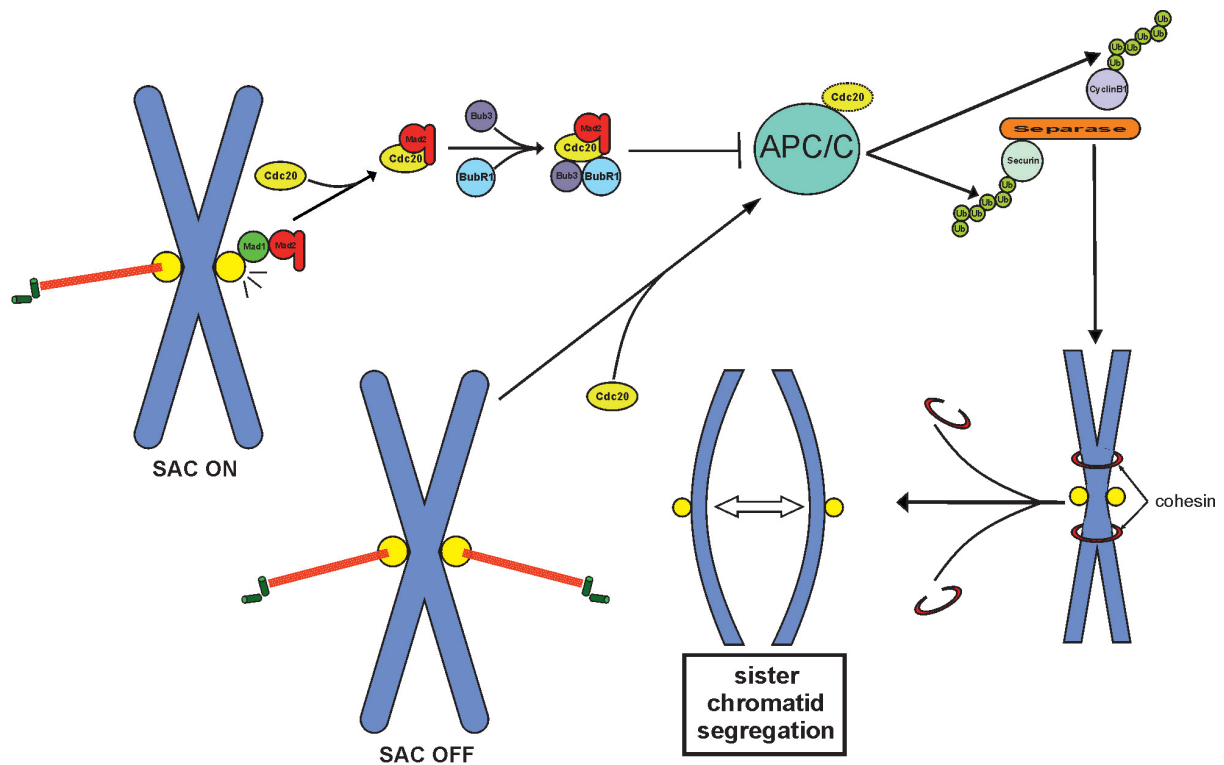
Main mechanisms that fulfil these criteria are the spindle assembly checkpoint and the resolution of non-amphitelic arrangements.

### **1.5.1. Spindle Assembly checkpoint and its major components**

The most important and sophisticated mechanism preventing premature sister chromatid separation is the spindle assembly checkpoint (SAC). The SAC involves more than a dozen components located at different positions in the nuclear area, ensuring sustained engagement of sister chromatids during prometaphase. The SAC has a high relevance in securing genomic integrity. As the process of sister chromatid segregation is irreversible, uncorrected spindle defects will ultimately enforce aneuploidy. This can have severe consequences and may lead to oncogenic transformation and cancer. It is well known that in most cancers the SAC is deregulated. Especially in breast cancer, SAC genes are commonly mutated or deregulated and the degree of deregulation correlates with the clinical outcome of the patients (Weaver and Cleveland, 2006; Yuan et al., 2006).

The SAC mechanism consists of several kinetochore-bound and soluble components. The SAC recognizes both unattached and improperly attached kinetochores and one single event is sufficient to initiate SAC signalling and halt mitotic progression. This is accomplished by Mad2 (**Mitotic arrest deficiency**), a small 24 kDa protein, capable of forming multimers and directly inhibiting APC/C coactivator Cdc20, thus preventing separase activity by ensuring sustained stability of its inhibitors cyclin B1 and securin. Binding of Mad2 to Cdc20 further recruits BubR1 and Bub3 to form the mitotic checkpoint complex (MCC). Importantly, the MCC can bind to the APC/C *in vitro* and *in vivo* but in this complex cannot serve as an activator for the APC/C to prematurely sequester cyclin B1 and securin (Sudakin et al., 2001). As long as the SAC is active, turnover of free Cdc20 into the MCC is performed (Fig. 3).

For studying the SAC, experimental approaches making use of microtubule drugs are extremely useful. Microtubule depolymerization drugs like nocodazole and microtubule polymerization drugs like taxol arrest mitotic cells at the prometaphase state. In the presence of nocodazole cells are incapable of assembling spindle microtubules. Consequently, the mitotic spindle cannot form. Taxol does not prevent microtubule polymerization but microtubule dynamics. Thus, the cell is incapable of generating correct tension force. As a consequence of the presence of microtubule drugs, cells exhibit sustained SAC activity.



**Fig. 3: Basic scheme of the spindle assembly checkpoint.** The checkpoint requires unattached or improperly attached kinetochores to be active (SAC ON) initiating binding of Mad2 to APC/C coactivator Cdc20, further recruiting BubR1 and Bub3. This complex is incapable of activating the APC/C. Upon anaphase onset and checkpoint silencing (SAC OFF), Cdc20 is no longer obstructed by Mad2. It can now serve as an activator of the APC/C, leading to ubiquitylation and destruction of the separase inhibitors securin and cyclin B1. Separase becomes active and cleaves centromeric cohesin. Sister chromatids are no longer engaged and separate towards opposite poles.

The essential target of the SAC is Cdc20. This protein of approx. 55 kDa can be roughly divided into two domains: A 175-aa N-terminal and a 325-aa C-terminal domain. The N-terminal domain harbours most phosphorylation and binding sites whereas the C-terminal domain is of structural importance (Fig. 4). It has been shown that Cdc20 is phosphorylated by Cdk1, MAPK and Bub1 kinases *in vitro* and *in vivo* (Wu et al., 2000; Tang et al., 2001; D'Angiolella et al., 2003). Phosphorylation of Cdc20 by Cdk1 seems to be necessary for its inhibition by the SAC: Cdk1-phosphorylation deficient Cdc20 greatly enhances *in vitro* activity of purified APC/C in comparison to wild-type Cdc20 (Yudkovsky et al., 2000). Furthermore, phosphorylation of Cdc20 is reported to be required for Mad2-dependent checkpoint activation as mitotic inhibition of Cdk1 triggers rapid Cdc20-Mad2 disassembly (Chung and Chen, 2003; D'Angiolella et al., 2003). Addi-

tionally, Bub1 phosphorylation of Cdc20 has been reported to have a negative impact on APC/C activity: Prometaphase arrested Hela cells stably expressing a Bub1-phosphorylation deficient Cdc20 mutant override the checkpoint and partially develop tetraploidy. In accordance, *in vitro* activity of purified APC/C<sup>Cdc20</sup> is strongly reduced after addition of recombinant Bub1 (Tang et al., 2004).

Cdc20 exhibits a multitude of different associations and intracellular localization patterns. During mitosis, Cdc20 is either free and persists as a monomer, associates with the MCC or binds to the APC/C. Furthermore, it has been shown that Cdc20 localizes to kinetochores in early prophase and shows the highest abundance at the metaphase-to-anaphase transition. Shortly after chromosome segregation, Cdc20 is removed from the kinetochores. How Cdc20 is recruited to the kinetochores and how localization affects the spindle assembly checkpoint remains elusive. It is suggested that kinetochoric localization of Cdc20 supports Mad2-dependent MCC formation (Kallio et al., 2002; Li et al., 2010).

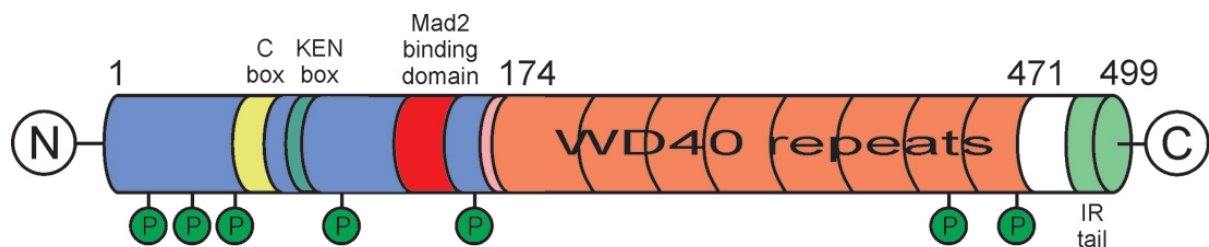
Cdc20 contains several domains required for proper checkpoint functionality and APC/C activity. The last two amino acids of Cdc20, isoleucine and arginine, known as the IR-tail, are necessary for binding both the APC/C subunit Cdc27 and MCC component BubR1 *in vitro* and *in vivo* (Passmore et al., 2003; Amador et al., 2007). Furthermore, it contains a distinct binding domain, the C-box at aa77-83, a consensus sequence found in all known APC/C co-activators, important for proper substrate recognition by the APC/C (Schwab et al., 2001). Another domain is the KEN-box, which is also located at the N-Terminus. It has been shown that the KEN-box is required for destruction of Cdc20 by the APC/C (Pfleger and Kirschner, 2000). It is not clear yet how destruction of Cdc20 contributes to SAC functionality or APC/C activity. Jonathan Pines and co-workers showed that Cdc20 is rapidly degraded in prometaphase arrested cells in the presence of translation inhibitor CHX and claim that continuous ubiquitylation by the APC/C followed by proteasomal destruction and re-expression of Cdc20 are required for maintenance of the SAC (Nilsson et al., 2008). On the other hand, Rape and colleagues claim that the (oligo-)ubiquitylation of Cdc20 triggers immediate disassembly of the MCC and checkpoint silencing (Reddy et al., 2007). However, this study was performed with an *in vitro* extract of mitotic Hela cells. Under native cellular conditions,



ubiquitylation of Cdc20 does not seem to drive disassembly of Mad2 (Mansfeld et al., 2011). Thus, the functional nature of Cdc20 ubiquitylation remains elusive.

An important issue of Cdc20 function is the fact that at least three different domains have been shown to interact with APC/C subunits: The IR-tail and the C-box mediate association required for proper APC/C functionality and substrate recognition. Furthermore, a third interaction seems to be essential for checkpoint maintenance: It has been shown that Cdc20 associates with the APC/C subunit APC8 when the SAC is active irrespective of the IR tail or the C-box. Upon checkpoint silencing this binding is abolished (Izawa and Pines, 2011). Interestingly, during the early stages of mitosis, Cdc20 cooperates with the APC/C to degrade cyclin A and Nek2A, indicating that the substrate specificity of the APC/C depends on different intramolecular APC/C-Cdc20 interactions (Geley et al., 2001). It has been shown that binding of Cdc20 to APC8 is essential for the ability of APC/C<sup>Cdc20</sup> to target SAC-insensitive proteins whereas binding of Cdc20 to both APC10 and Cdc27 is needed for efficient ubiquitylation and degradation of cyclin B1 and securin (Izawa and Pines, 2011).

Of structural importance are the WD-40 repeats within the C-terminal region of Cdc20. This domain consists of seven repeated structural motifs forming a beta-propeller, required for proper interaction with various proteins and substrate recognition (Yu, 2007). The nature of the WD40 repeats are still under investigation. It has been stated that this domain is required for proper localization to kinetochores during mitosis in human cells whereas in *D. melanogaster* it seems that BubR1 directs Cdc20 to the kinetochores (Kallio et al., 2002; Li et al., 2010).



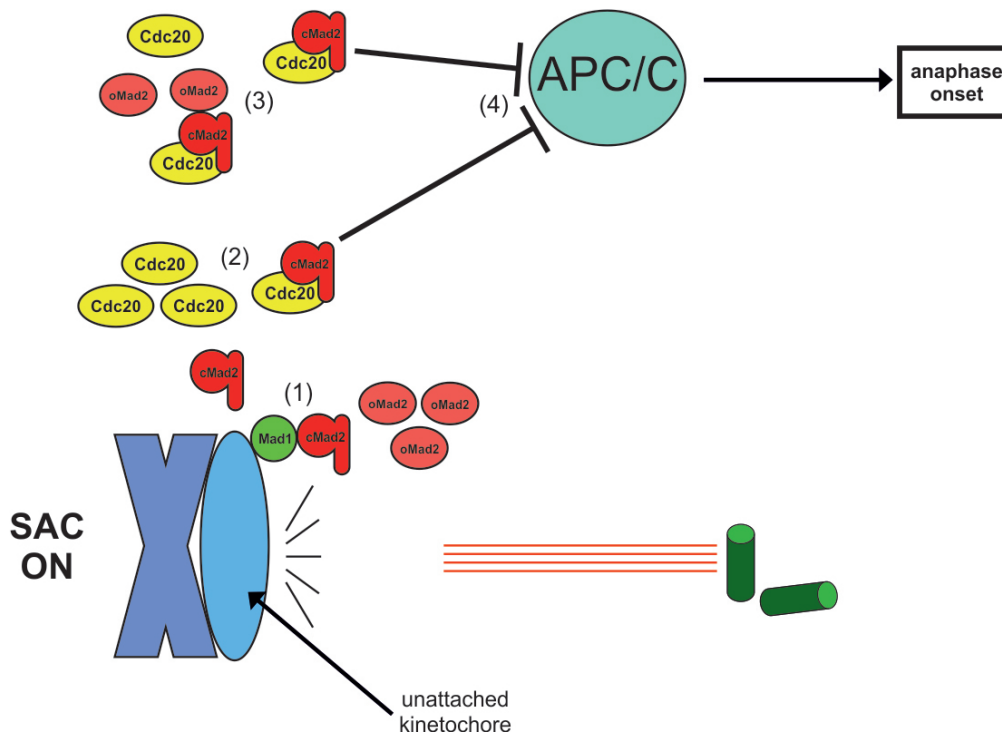
**Fig. 4: Basic structure of human Cdc20:** Cdc20 can roughly be divided into two domains: The 174-aa N-terminal domain harbours most of the Cdk1, Bub1 and MAPK phosphorylation sites, destruction boxes and the Mad2 binding site. The 325-aa C-terminal domain harbours the WD40 repeats and the IR tail. The green circles indicate Cdk1-phosphorylation sites. See text for details.



### **1.5.2. *The template model***

How can the cell enable the rapid turnover of all present Cdc20 species into MCCs, even if only one chromosome is not or improperly attached to the mitotic spindle?

The template model involves two essential components of the SAC, the Mad proteins Mad1 and Mad2. Mad1 is localized at the outer kinetochore and its accurate localization requires CenpE and Mps1-directed phosphorylation. (Liu et al., 2003). Furthermore, Mad1 recruitment depends on a non-catalytic activity of Bub1 kinase (Sharp-Baker and Chen, 2001). The presence of Mad1 at unattached kinetochores fundamentally recruits Mad2. Interestingly, Mad2 can adopt two different conformations dependent on the current status of the spindle assembly checkpoint. The open conformation (oMad2) refers to soluble Mad2 in its inactive form. The closed conformation (cMad2) allows the protein to create a “safety belt”, enabling stable binding to APC/C-coactivator Cdc20 and the formation of an inhibitory complex (Chen et al., 1996; Luo et al., 2004). It has been suggested that during active checkpoint signalling, kinetochore bound Mad1 recruits oMad2, initiating its transformation. cMad2 itself can alter the conformational state of oMad2 species to their closed conformation. This launches a rapid cascade leading to fast and complete Mad2-dependent inhibition of Cdc20. Hence, kinetochoric Mad1 acts as a catalytic unit to stimulate Cdc20-Mad2 formation explaining why only one unattached kinetochore is sufficient to generate the “wait anaphase signal” (see Fig. 5). The whole process of rapid checkpoint activation is still under investigation: Recently, it has been shown that the nuclear protein RED is required for proper recruitment of Mad1 to the kinetochores (Yeh et al., 2012).



**Fig. 5: The Mad1/Mad2 template model:** Mad1/Mad2-dependent inhibition of Cdc20 is initiated by unattached or improperly attached kinetochores, leading to the recruitment of major elements of the SAC signalling pathway. **(1)** Association of kinetochoric Mad1 with open Mad2 (oMad2) induces conformational alteration to closed Mad2 (cMad2), which has a high affinity for free Cdc20 **(2)**. Furthermore cMad2 initiates a conformational change of soluble oMad2 to cMad2, thus triggering rapid distribution of the “wait anaphase”-signal **(3)**. Inhibited by Mad2, Cdc20 cannot serve as a co-activator of the APC/C **(4)**. See text for details.

### 1.5.3. SAC silencing

After all chromosomes are properly aligned to the mitotic spindle, the SAC has to be deactivated (silenced) for the APC/C to become active and trigger anaphase. It is not yet fully understood which mechanisms lead to SAC silencing and there are several contradictory views in the field. As discussed earlier in this work, it has been proposed that APC/C-dependent (oligo)ubiquitylation of Cdc20 induces rapid disassociation of Mad2 from Cdc20. Without its inhibition within the MCC, Cdc20 can then function as a co-activator of the APC/C (Reddy et al., 2007). However, a growing body of evidence suggests that there is no connection between Cdc20 ubiquitylation and checkpoint deactivation. Jonathan Pines and co-workers report that Mad2 still disassociates in a timely manner from a non-ubiquitinatable Cdc20-variant during metaphase, although it has to be mentioned that cells expressing this variant overcome the checkpoint prematurely in nocodazole or taxol arrested cells. (Nilsson et al., 2008; Mansfeld et al., 2011). A proven

role in rapid silencing is fulfilled by the Mad2-binding protein p31<sup>Comet</sup>, which prevents cMad2 from generating inhibitory complexes with Cdc20. As a result, Cdc20 can no longer be sequestered into the MCC. (Xia et al., 2004; Teichner et al., 2011).

Furthermore it is hypothesized, that rapid silencing might additionally depend on a short half-life of inhibitory MCCs in general, indicating that sustained SAC activity requires continuous Cdc20 turnover. Upon p31<sup>comet</sup>-dependent inactivation of Mad2, the “wait anaphase”-signal would then rapidly vanish (Hagan et al., 2011). However, the exact mechanism of checkpoint silencing still remains elusive.

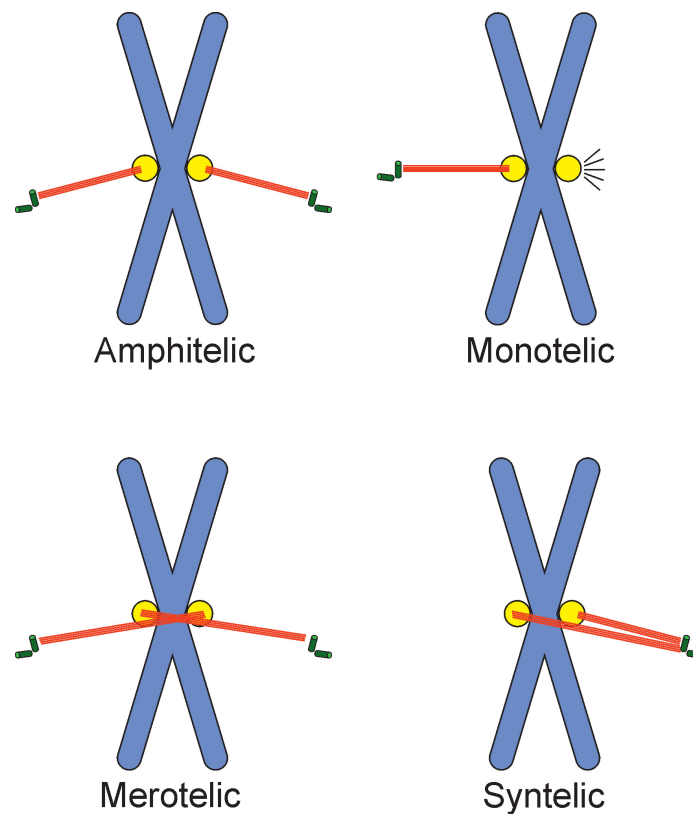
#### ***1.5.4. Resolution of syntelic and merotelic attachments***

Besides correct amphitelic attachments, there are several ways that microtubules can inappropriately attach to the kinetochores (Fig. 6). These mis-arrangements have to be resolved before anaphase onset. However, only unattached kinetochores (monotelic attachment) or insufficient arm tension are able to initiate a recruiting cascade leading to activation of the SAC. For that reason the cell exhibits a number of SAC-independent mechanisms ensuring proper alignment of the chromosomes to the mitotic spindle.

Syntelic attachments are mainly resolved by the mitotic kinase aurora B. During prometaphase, this kinase accumulates at kinetochores, phosphorylating components of the Ndc80 complex amongst others (Knowlton et al., 2006). It has been shown that inhibition of aurora B kinase during prometaphase leads to a severe increase in unresolved syntelic attachments (Cheeseman et al., 2006; DeLuca et al., 2006). It is hypothesized that aurora B recognizes kinetochores which are not under tension, as only bi-oriented, amphitelic attachments generate correct tension forces. By phosphorylating several components, kinetochore-microtubule bindings are probably weakened.

Since merotelic attachments involve both sister chromatids and therefore do not generate SAC signals via unattached kinetochores, they most likely also establish correct tension force. It is as yet unclear how these mis-arrangements are resolved. It has been shown that aurora B kinase is also enriched near of merotelic attachments (Knowlton et al., 2006), however the mechanism has to differentiate from those leading to resolution of syntelic attachments. It is also thinkable that some merotelic attachments are resolved intentionally before and during anaphase without any specific components be-

ing required. Importantly, merotelic attachments are a great cause of aneuploidy and the primary cause of genomic instability in cancer, indicating that cells might lack efficient strategies to resolve merotelic attachments (Ruchaud et al., 2007; Gregan et al., 2011).



**Fig. 6: Chromosome attachment to the mitotic spindle:** In addition to correct and faithful bipolar spindle attachment (amphitelic), the cell can be confronted with different possibilities of incorrectly attached mitotic spindles or attachments which do not resemble correct spindle force. Monotelic chromosomes contain one unattached kinetochore and activate the SAC. Merotelic attachments contain attached microtubules connected to the opposite poles and generate correct spindle force. In the case of syntelic attachments both kinetochores are connected to the same spindle pole.

## 1.6. Sister chromatid segregation

### 1.6.1. The E3 ubiquitin ligase APC/C

In anaphase, sister chromatids are irreversibly segregated from each other. Before separate ultimately triggers this separation process, its inhibitors securin and cyclin B1 are covalently linked with ubiquitin chains, leading to their degradation by the 26S-

proteasome. This pathway involves the anaphase promoting complex or cyclosome (APC/C), a giant 1,5 MDa multiprotein complex acting as an E3 ubiquitin ligase. The APC/C plays a fundamental role in the metaphase-to-anaphase transition (Peters, 2002).

The APC/C and its collaborators are thoroughly regulated. Upon activation of ubiquitin by the E1 activating enzyme, the APC/C requires both Ubch5 and Ubch10 E2 conjugation enzymes to fulfil its complete functionality *in vivo* (Rodrigo-Brenni and Morgan, 2007). *In vitro*, however, either Ubch10 or Ubch5 seem to be sufficient to support full APC/C activity (Summers et al., 2008). Interestingly it has been shown that Ubch10 specifically interacts with the APC/C, but concerning Ubch5, various E3 enzymes have been identified as a putative collaborator (Kim et al., 2007). Although a three-dimensional structure of the APC/C is available, it is not yet fully understood how ubiquitin chains are transferred to substrates and how collaboration between the APC/C and its regulators and substrates works. It has been proposed that the APC/C subunits build a clamp making room for a coactivator to position the substrate in closed vicinity of the E2 enzyme (Fig. 7). For the APC/C subunits APC2, APC10 and APC11, a direct involvement in this catalytic event has been proven (da Fonseca et al., 2011).

In vertebrates the APC/C consists of 13 known core subcomponents, most of them having homologs in yeast. Recently APC15 was identified as a new subunit of the APC/C, necessary for continuous turnover of the MCC during SAC signalling, proving that the exact APC/C composition is still under investigation (Mansfeld et al., 2011).

In mammalian cells two co-activators of the APC/C have been identified so far, namely Cdc20 and Cdh1. Homologues of these proteins have been found in all analyzed eukaryotes. In yeast, Ama1, a meiosis-specific activator has been identified and is essential for the first meiotic division and sporulation (Cooper et al., 2000). Until now, searches for a homologue of Ama1 in mammals have not been successful.

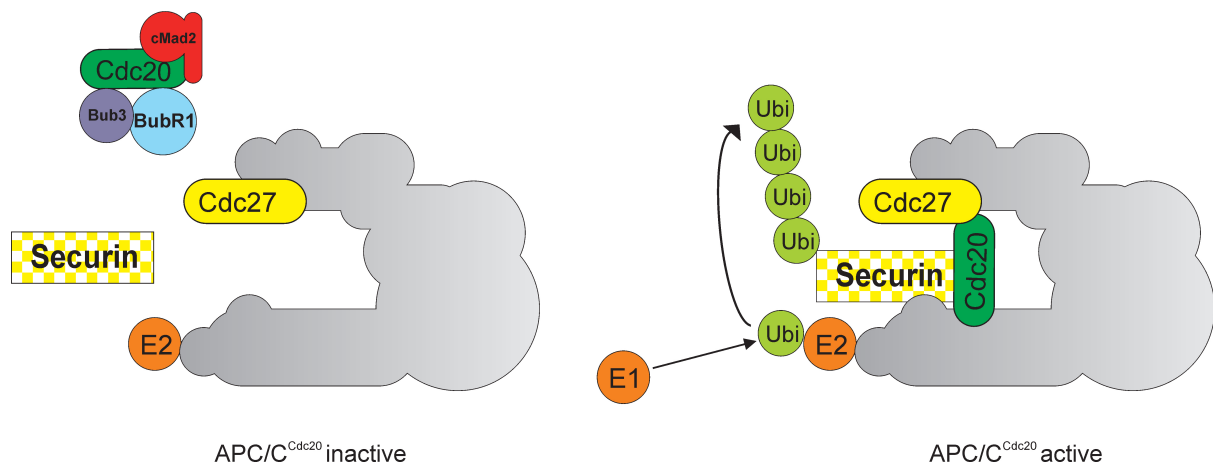
Cdc20, Cdh1 and Ama1 have distinct domains in common: The C-box, the WD-40 repeats and the last two amino acids, known as the IR-tail, which mediates binding to Cdc27 (Pesin and Orr-Weaver, 2008). Although only three APC/C activators are known up to now, the list of known APC/C inhibitors contains more than 20 members. Important for timely regulation of the APC/C during mitosis are the components of the MCC,

Mad2 and BubR1, which inhibit Cdc20 before anaphase onset. Kinases involved in SAC signalling like Bub1, MAPK and Cdk1 inhibit the APC/C via Cdc20 phosphorylation (Peters, 2006). Another example is Mnd2 which serves as an inhibitor and prevents precocious securin degradation during yeast meiosis (Oelschlaegel et al., 2005). In *Xenopus laevis*, the stoichiometric APC/C inhibitor XErp1 is of importance for establishing a metaphase arrest (CSF arrest) during meiosis II. Upon fertilization XErp1 is rapidly degraded, leading to activation of the APC/C and anaphase onset (Tung et al., 2005).

The APC/C shows a differential localization pattern during mitosis. Besides being present in the soluble nucleoplasmatic fraction, localization can also be observed in centrosomes and at the kinetochores. Furthermore, microtubule-association has been described (Tugendreich et al., 1995, 1995; Torres et al., 2010).

An important issue in APC/C biochemistry is regulation via phosphorylation: Not only are the APC/C co-activators Cdc20 and Cdh1 targeted for inhibitory phosphorylation by various kinases, but efficient substrate recognition also requires phosphorylation of the substrate (Peters, 2006). Furthermore, several phosphorylation sites within APC/C subunits have been identified: The APC/C subunit Cdc27 is phosphorylated upon entry into mitosis even before kinetochore assembly and checkpoint activation (Huang et al., 2007). This phosphorylation seems to have an essential role in regulating the APC/C toward the TGF- $\beta$  signalling pathway (Zhang et al., 2011).

There is a growing body of evidence claiming the importance of acetylation of the APC/C and its regulators for mitotic progression. It has been suggested that BubR1 acetylation triggers its APC/C-dependent degradation, promoting APC/C<sup>Cdc20</sup> activity and anaphase onset (Choi et al., 2009).



**Fig. 7: The APC/C before and at anaphase onset:** Degradation of securin/cyclin B1 during metaphase/anaphase requires APC/C-coactivator Cdc20, which is inhibited by the MCC during SAC signaling (APC/C<sup>Cdc20</sup> inactive). Upon anaphase onset, Cdc20 is used as a molecular link inside the clamp of the APC/C in close proximity to the E2, which collaborates with the E1 in physically connecting securin and cyclinB1 with ubiquitin chains (APC/C<sup>Cdc20</sup> active).

### 1.6.2. The ubiquitin proteasome system

Temporal ubiquitylation and proteasomal degradation of cyclin B1, securin and other targets is essential for mitotic progression (Murray et al., 1989; Cohen-Fix et al., 1996; Bazile et al., 2008). Most of the ubiquitylated proteins are efficiently targeted to the proteasome and finally cleaved although it has to be stated that mitotic regulation also makes use of non-proteolytic ubiquitylation (Hörmanseder et al., 2011). Ubiquitylation is performed by a subset of various proteins, namely E1, E2 and E3 enzymes. E1 or ubiquitin-activating enzymes form a thioester bond with the C-terminal glycine of a ubiquitin molecule, transferring it to a ubiquitin conjugating enzyme or E2, which collaborates with an E3 ubiquitin ligase to promote covalent transfer of the ubiquitin to an epsilon-amino group of the target substrate. This ATP-dependent reaction is progressive and leads to oligo- or polyubiquitylation of the substrate as ubiquitin molecules are continuously added to persisting ubiquitins (Reed, 2006).

Proteasomes are large proteinaceous cell organelles and present in all eukaryotes and members of the kingdom of *Archaea*. In higher eukaryotes they are present in the cytosol and the nucleoplasm. They have a size of 26S containing a core subunit of 20S and two regulatory subunits of 19S each. The assembly of proteasomes is a complicated, not yet fully understood process of post-translational modifications and self-cleavage being

involved (Kikuchi et al., 2010). Proteolysis is performed inside the 20S core complex and involves several catalytic subunits, each having a preference for special motifs inside the substrates (Heinemeyer et al., 1997). The proteasome pathway also plays a role in non-ubiquitylation driven post-translational processing of proteins, especially of some transcription factors (Palombella et al., 1994).

### **1.6.3. Cohesin**

Cohesin acts as an essential complex in chromosome and centrosome segregation during mitosis and meiosis. It consists of four subunits: Smc1, Smc3, Scc3 and the  $\alpha$ -kleisin subunit Scc1/Rad21, which is exchanged for the homologous protein Rec8 in meiosis. Both Scc1 and Rec8 share common motifs that are recognized by separase prior to anaphase. Recently, a new  $\alpha$ -kleisin subunit has been identified: Rad21L (Rad21 like protein) plays a role in early meiosis and is conserved in vertebrates (Lee and Hirano, 2011).

There is meaningful evidence that the four subunits of cohesin form a topological ring which entraps the DNA stands of both sister chromatids: Cleavage of either DNA or cohesin in artificial circular minichromosomes purified from yeast cells abolishes DNA-cohesin association (Haering et al., 2002; Ivanov and Nasmyth, 2005).

In animal cells, cohesin loading is established during S-phase and most cohesin rings are removed during prophase by Plk1-driven phosphorylation of Scc3 (Shen et al., 1998). However, centromeric cohesin is protected from this non-proteolytical mechanism and sustained until anaphase. Resistance against premature dissolution of cohesin is accomplished by the centromeric presence of so called shugoshins. So far two members of the shugoshin family have been detected in mammalian cells, named Sgo1 and Sgo2 (Marston et al., 2004). In growing mitotic cells, Sgo1 prevents premature sister chromatid disengagement: Repression of Sgo1 causes loss of centromeric cohesin prior to anaphase onset (McGuinness et al., 2005). It has been suggested that Sgo1 prevents cohesin from being phosphorylated by Plk1 rather than protecting against proteolysis by separase as Scc1 is timely cleaved at the metaphase-to-anaphase transition. Further strengthening this point is the finding that protein phosphatase PP2A associates with



Sgo1 at centromeres, counteracting Scc3 phosphorylation, thus preventing premature loss of cohesion (Kitajima et al., 2006).

#### **1.6.4. Separase**

After all kinetochores are properly aligned at the spindle apparatus and the SAC is satisfied, separase finally triggers disengagement of the entrapped sister chromatids. Separase (Esp1 in yeast) is a cysteine protease of 233 kDa (in humans) and responsible for cleaving centromeric Scc1/Rad21 in mitosis and Rec8 in meiosis, therefore acting as the direct trigger for anaphase onset. Furthermore, separase is also directly involved in centriole separation by cleavage of centrosomal cohesin (Schöckel et al., 2011). Separase was originally identified in yeast cells, where temperature-sensitive variants show perturbed spindle pole regulation (Baum et al., 1988). However, several studies also indicated a role of separase in sister chromatid separation as Esp1 mutant yeast cells exhibited a severe reduction in the separation of centromeric sequences (Ciosk et al., 1998a; Uhlmann et al., 1999).

Premature cleavage of cohesin during mitosis has severe consequences, therefore separase function is chiefly regulated by at least two inhibitory mechanisms: It has been shown that upon mitotic entry, separase is phosphorylated by Cdk1/cyclin B1 on several sites, one of them being the serine residue 1126. This phosphorylation mediates Cdk1/cyclin B1 dependent binding and inhibition (Stemmann et al., 2001; Gorr et al., 2005). Additionally, securin, the second known stoichiometric inhibitor can associate with separase in a mutually exclusive manner (Ciosk et al., 1998b; Stemmann et al., 2001) and also acts as a chaperone for separase (LeBrasseur, 2002). Interestingly, HCT116 cells lacking securin show normal chromosome segregation behaviour and homozygous securin knockout mice are viable (Wang et al., 2001; Pflieger et al., 2005), indicating that there might be additional regulatory mechanisms, that are yet to be discovered. Besides cohesin, separase also promotes its autoproteolytic cleavage at several distinct sites. This mechanism seems to be indispensable for proper separase function as the prevention of autocleavage induces abnormal spindle assembly effects and prolonged mitotic duration (Papi et al., 2005).

## 1.7. The prolyl isomerase Pin1

### 1.7.1. Structure and basic functions of Pin1

The crucial cis/trans prolyl isomerase Pin1 (**P**eptidylprolyl cis/trans isomerase, **N**IMA-interacting 1) is a protein of 17 kDa involved in various cellular processes. Initially it was identified in a screen for mitotic proteins interacting with NIMA kinase in *Aspergillus nidulans* (Lu et al., 1996). Basically, Pin1 mediates cis/trans isomerisation of its substrates and induces a conformational change. This catalysis is neither energy-driven nor ATP-dependent and does not require additional cofactors. Furthermore this conformational switch is of a reversible nature (Lu and Zhou, 2007).

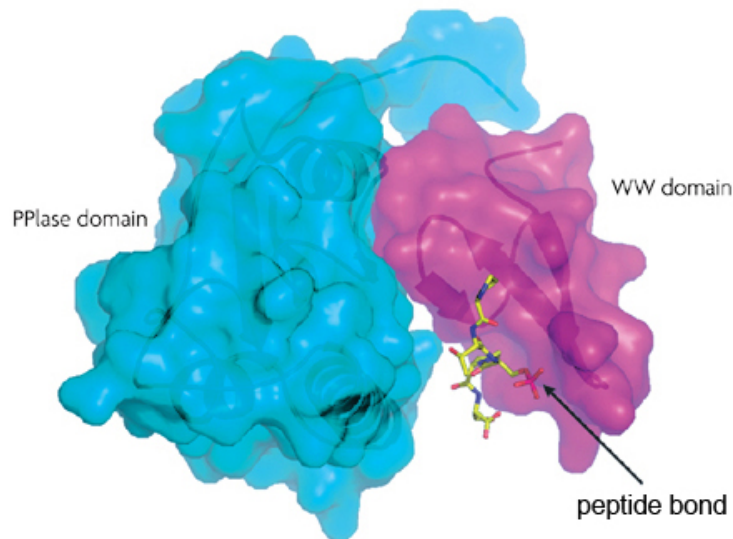
Pin1 consists of two distinct domains: The N-terminal 34 aa WW-domain is pivotal for substrate recognition and recruits them to the nearby PPlase domain, which then performs the cis/trans-isomerization (Lu et al., 1996; Fig. 8). Pin1 specifically recognizes phospho-serine/threonine-proline motifs *in vitro* and *in vivo* and therefore acts as a phospho-specific isomerase (Shen et al., 1998). It has been shown that Pin1 is present in most human cells and expression levels are unaltered throughout the cell cycle. However, Pin1 shows a preference for mitotic rather than for interphase substrates. Pin1 mainly localizes to the nucleus with a low-level presence in the cytoplasm. Interestingly, Pin1 does not contain any NLS or NES sequences and translocation to the nucleus is probably mediated via its substrates (Shen et al., 1998; Bao et al., 2004; Rippmann et al., 2000).

Being a crucial enzyme involved in many cellular processes, Pin1 itself is target of regulatory mechanisms: It is phosphorylated by Plk1 and PKA. Phosphorylation by PKA on serine residue 16 of Pin1 mediates immediate loss of substrate binding capability (Lu et al., 2002). Furthermore it has been shown that Plk1-directed phosphorylation of Pin1 on serine residue 65 prevents its ubiquitylation and thus provides its stability during mitotic progression (Eckerdt et al., 2005).

How do Pin1-induced conformational switches alter substrate properties? Up to now, more than two dozen Pin1 substrates have been identified and consequences of Pin1-mediated isomerization are very manifold. Pin1 can influence the degree of ubiquitylation of certain substrates: It is known that Pin1 prevents polyubiquitylation of p53, sub-

sequently promoting its stabilization (Siepe and Jentsch, 2009). On the other hand, Pin1 can also enforce ubiquitylation and degradation as it has been shown for cyclin E (Yeh et al., 2006). Pin1 enhances phosphorylation of topoisomerase II $\alpha$ , which is required for chromosome condensation. Interestingly Pin1 shows enhanced chromosomal localization during G2/M phase, making it likely that Pin1 promotes proper functionality of chromosome-bound phosphoproteins (Xu and Manley, 2007). Pin1 is also indispensable for proper subcellular localization of cyclin D1 and NF- $\kappa$ B, underlining Pin1's significance in cell cycle progression and transcription regulation (Liou et al., 2002; Ryo et al., 2003).

Surprisingly, homozygous Pin1<sup>-/-</sup> mice are viable and fertile. The only observable defect is the incapability of Pin1<sup>-/-</sup> mouse embryonic fibroblasts to re-enter G1 after a prolonged G0 arrest (Fujimori et al., 1999). This occurrence indicates that some Pin1 functions can be executed by other prolyl isomerases. Another interesting aspect is that yeast cells expressing Pin1 at a very low level of 5 % compared to normal abundance, do not show any invariable phenotype, indicating that low levels of Pin1 activity seem to be sufficient to reliably control Pin1-dependent switches (Gemmell et al., 2005).



**Fig. 8. Basic structure of Pin1:** This small protein with a molecular weight of 17 kDa contains a WW domain (shown in red) required for recognition of phospho-Ser/Thr-Pro motifs and a catalytic PPlase domain (shown in blue) necessary for isomerization of the peptide bond within the substrate, which is highlighted by an arrow. Figure taken and modified from (Lu and Zhou, 2007).

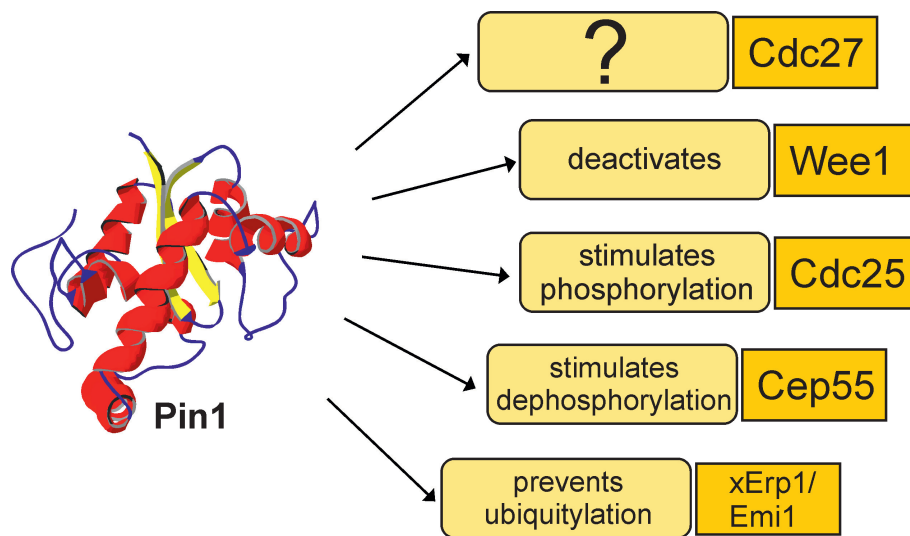
### **1.7.2. Mitotic and cell cycle related functions of Pin1**

Pin1 is of major importance for proper mitotic entry: On the one hand, data from experiments strongly suggests that Pin1 binds to Wee1 kinase, leading to its enzymatic inactivation. Consequently, Cdc25-directed dephosphorylation of Cdk1 on residues Tyrosine 14 and Threonine 15 cannot be counteracted by Wee1 anymore, enabling high Cdk1 activity (Okamoto and Sagata, 2007). On the other hand, it is known that Pin1 also directly inhibits Cdc25 activity, indicating that Pin1 regulates Cdk1 dephosphorylation by at least two different ways (Stukenberg and Kirschner, 2001). Surprisingly, experiments show that overexpression of Pin1 in Hela cells or addition of high amounts of the corresponding recombinant protein to *X. laevis* interphase extract efficiently blocks mitotic entry via a G2 arrest (Lu et al., 1996; Crenshaw et al., 1998), indicating that Pin1 harbours additional functions in regulating entry into mitosis. This G2 arrest might be explained by Pin1's influence on APC/C inhibitor Emi1, which is stabilized by Pin1 through preventing its SCF-dependent degradation (Bernis et al., 2007). It is also known that Pin1 binds to APC/C component APC3/Cdc27 during mitosis, presumably in a Cdk1 phosphorylation depended manner. However, there are no indications so far as to how Pin1/Cdc27 interaction might alter Cdc27 or APC/C function (Shen et al., 1998). As Cdk1-directed phosphorylation of Cdc27 seems to be pivotal for the proper chromosomal localization of Cdc27 in *D. melanogaster* embryos, it is possible that Pin1 stimulates this localization process (Huang et al., 2007). Another known mitotic function of Pin1 is the involvement in the regulation of cytokinesis. Pin1 localizes to the midbody ring during cytokinesis and directly binds to Cep55 promoting its Plk1-directed phosphorylation (van der Horst and Khanna, 2009). Furthermore, there is evidence that Pin1 indirectly contributes to the duration of the spindle assembly checkpoint in prometaphase arrested cells: Nocodazole arrested Hela cells expressing a Pin1-binding deficient variant of the nucleotide exchange factor Sil1 deactivate the SAC and cycle back into G2 phase (Campaner et al., 2005). However, a direct involvement of Pin1 in SAC functionality could not be shown yet.

A summary of some cell cycle related Pin1 features is shown in Fig. 9.

Although Pin1 primarily associates with mitotic phosphoproteins, several Pin1-directed processes during interphase have been investigated, among them being inflammation,

immune response and embryonic development (Esnault et al., 2007; Wildemann et al., 2007; Liu, 2009). It has been shown that Pin1 prevents apoptosis during interphase by maintaining levels of Mcl1, an anti-apoptotic protein. (Becker and Bonni, 2007). In accordance with being required for mitotic entry, Pin1 is also involved in S-Phase entry by stabilizing cyclin D1 (Wulf et al., 2001).



**Fig. 9: Mitotic or cell cycle related functions of Pin1:** Pin1 influences several cell cycle-dependent mechanisms: It is essential for faithful entry into mitosis by dephosphorylating Cdc25 phosphatase and deactivating Cdk1 inhibitor Wee1. Furthermore, Pin1 stimulates the Plk1-driven phosphorylation of Cep55 required for its proper localization to the midzone. Pin1 has been shown to stabilize APC/C inhibitor Emi1 during G2 phase. See text for details.

### 1.7.3. Involvement of Pin1 in diseases

There have been several studies linking Pin1 to a multitude of diseases, including cancer and Alzheimer's Disease: Pin1 is overexpressed in several breast cancer tissues and the degree of Pin1 expression correlates with the clinical outcome of patients (Wulf et al., 2001; Lam et al., 2008). Moreover, a pathological correlation between Pin1 upregulation in prostate cancer cells and the clinical stage of the tumour could be proven (Ayala et al., 2003). In several other cancer types such as colon and melanoma cancer, a strong Pin1-overexpression was found (Bao et al., 2004). One possible explanation for a pro-oncogenic role of Pin1 is that in some breast cancer cells, Pin1 cooperates with a mu-

tated form of p53 protein to promote transcriptional activation of oncogenes (Girardini et al., 2011). Supportingly, knockout of Pin1 in prostate cancer cells suppresses cell proliferation and other tumorigenic features like cell migration and invasion (Ryo et al., 2005). Based on these data, Pin1 could be classified as an oncogene, although data that had been published suggests the contrary: Pin1 stabilizes the oncosuppressors p53 and p73 by preventing their poly-ubiquitylation and proteasomal degradation (Wulf et al., 2001, 2002; Siepe and Jentsch, 2009). These observations make it difficult to stigmatize Pin1 as a crucial factor involved in tumour biology, as Pin1 seems to either suppress or promote tumour progression.

There are several clues, that downregulation of Pin1 is linked to neuronal degeneration in Alzheimer's Disease: It has been shown that Tau, a microtubule associated protein, is hyperphosphorylated and strongly enriched in accumulated intracellular neurofibrillary tangles (Grundke-Iqbal et al., 1986; Butterfield et al., 2006). Pin1 depletion or its subsequent enhances accumulation of phosphorylated Tau, causing neurodegeneration due to upregulation of phosphoproteins like Bcl-1 (Pathan et al., 2001; Butterfield et al., 2006).

Furthermore, Pin1 is also a factor involved in infection diseases: It is a prerequisite for proper replication of the HI-virus type I and also required for an efficient Hepatitis B life cycle (Watashi et al., 2008; Lim et al., 2011).

Together, these findings demonstrate an involvement of Pin1 in a number of various diseases. The pathological significance of Pin1 expression proposes the development of specific drugs that inhibit Pin1 (Xu and Etzkorn, 2009).

### **1.8. Special characteristics of meiosis**

Meiosis is a special form of cell division present in almost all eukaryotes. During meiosis, chromosomes are the targets of recombination events leading to new gene rearrangements. DNA segregation takes place twice: In a first round homologous chromosomes are segregated from each other and in a second round sister chromatids separate. Each round is terminated by cytokinesis. In the case of male mammalian meiosis, this will result in the production of four haploid spermatids. In the case of female meiosis, usually only one haploid cell (oocyte) is produced due to the asymmetric nature of

female cell division, resulting in polar body extrusion after each meiotic round (Morgan, 2006; Sun and Kim, 2012). Like in mitosis, the SAC also fulfils an important role in securing genomic integrity during meiosis. Segregation errors during meiosis will ultimately lead to aneuploidy, which is the major cause of miscarriages and birth defects, like trisomy 21. Many checkpoint components of the mitotic SAC, such as Cdc20, Mad2 and Mad1, have also been identified in the meiotic SAC signalling pathway. However, the molecular mechanisms involved in the meiotic SAC are poorly understood (Jin et al., 2010; Sun and Kim, 2012).

### **1.9. The African clawed frog as a suitable model organism for studying the cell cycle**

The cell free extract of unfertilized eggs (CSF extract) from the African clawed frog (*Xenopus laevis*) provides an *in vitro* system for biochemical and cell biological experiments and is especially suitable for studying cell cycle regulation. The CSF extract is made by high-speed centrifugation of non-fertilized eggs from hCG-injected female frogs. Despite being cell free, the CSF extract is arrested, like the unfertilized eggs, in metaphase II of meiosis. This is due to the activity of xErp1, an APC/C inhibitor preventing anaphase onset (Haccard et al., 1993). By adding  $\text{Ca}^{2+}$ , fertilization is mimicked and the extract exits metaphase/mitosis and enters interphase. The CSF extract is suitable for adding recombinant proteins or their corresponding mRNA and removing endogenous proteins by immunodepletion. As in cell culture systems, inhibitors can be applied and by addition of sperm and fluorophore-tagged microtubules to the extract, spindle assembly can be studied biochemically and microscopically. Moreover, regulation of the SAC can be investigated by adding high amounts of *Xenopus* sperm nuclei and nocodazole to the CSF extract, mimicking failed kinetochore-microtubule attachment, thus activating the SAC.

### **1.10. Aim of this work**

While performing experiments with a cell line inducibly expressing a phosphovariant of separase, former Ph.D. student Dominik Boos observed a reduction in the amounts of phosphorylated histone H3 in nocodazole arrested cells upon addition of Pin1 inhibitor

Juglone (data not shown). Pin1 is known as a molecular timer, being involved in a broad range of mitotic events, among them being proper entry in and exit from mitosis (Stukenberg and Kirschner, 2001; van der Horst and Khanna, 2009). The reduction of phosphorylated histon H3 raised the question whether Pin1 is involved in the spindle assembly checkpoint (SAC), as this phosphorylation is usually maintained upon nocodazole treatment. To answer this question, initial experiments in cell free extracts of *X. laevis* eggs (CSF extract) were performed. An antibody was raised against *X. laevis* Pin1 and used for its immunodepletion from the extract. After addition of high amounts of sperm nuclei and nocodazole to activate the SAC, the depleted extract was released from the CSF arrest. It was investigated whether securin was degraded under these conditions, indicating checkpoint override.

During the time of this thesis the specific chemical Pin1 inhibitor DTM was described, making it more suitable than Juglone, which is unspecific and general inhibits prolyl isomerases (Tatara et al., 2009). The new inhibitor was used in preliminary cell culture experiments, elucidating a possible checkpoint override upon Pin1 inhibition.

Indeed, preliminary results from both the *X. laevis* extract system and mammalian cell culture experiments indicated a role for Pin1 in the maintenance of the SAC. Encouraged by these data, it was decided to further investigate Pin1's role in SAC signalling.



## 2. RESULTS

### 2.1. Analysis of a putative role of Pin1 in the spindle assembly checkpoint

Motivated by promising preliminary results of former PhD student Dominik Boos, initial experiments were performed pursuing the question of whether Pin1 is involved in SAC signalling. However, on the one hand no suitable chemical inhibitor for use in research was available at the beginning of this study. Pin1-inhibitors such as juglone are too unspecific and generally inhibit prolyl isomerases (Hennig et al., 1998), thus influencing a multitude of cellular functions at the same time. On the other hand, Pin1 has several reported functions, which is why gradual depletion of Pin1 by RNAi would cause arrests at multiple cell cycle positions and therefore, would be inappropriate to selectively study possible SAC defects. With the newly described chemical Pin1 inhibitor dipentamethylene thiuram monosulfide (DTM), which was shown to be highly specific in disturbing catalytic activity and substrate recognition and is effective in relatively low concentrations in HCT116 cells (Tatara et al., 2009), a new perspective appeared. Now it became suddenly possible to instantaneously inhibit Pin1 after cells had synchronically entered mitosis and arrested at prometaphase due to SAC activation.

Another possibility to investigate the SAC is the use of cell free extracts of *Xenopus laevis* eggs (CSF extracts). As described in section 1.9 the CSF extract is arrested in metaphase of meiosis II and the SAC can easily be established by supplementing the extract with high amounts of sperm nuclei and the microtubule depolymerizing drug nocodazole. All components required for accurate SAC signalling are present within the extract. For studying the SAC in *X. laevis* a suitable antibody against xPin1 had to be raised. Both the antibody and the inhibitor had to be checked for their experimental suitability.

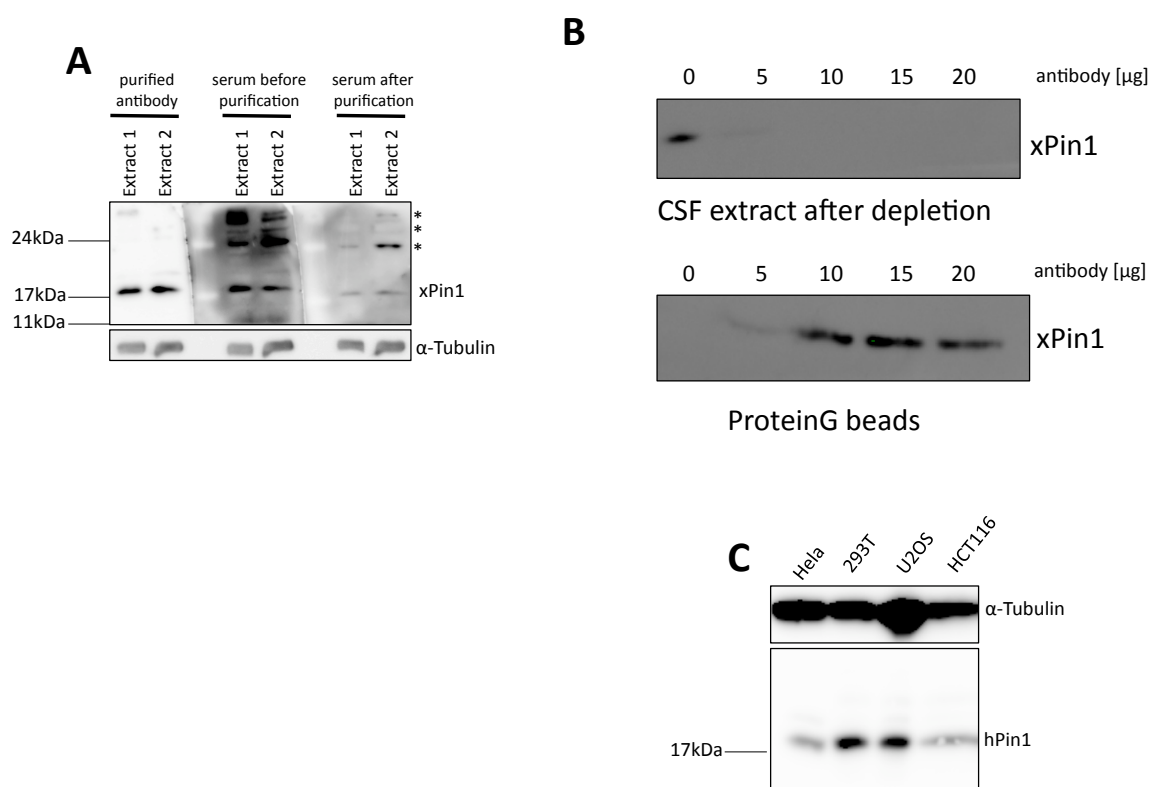
### 2.1.1. Characterization of tools for the study of Pin1

Studying the role of Pin1 in *Xenopus* egg extract required the generation of a suitable antibody for immunodepletion experiments. To this end, a polyclonal antibody against bacterially expressed and full-length *X. laevis* Pin1 (xPin1) was raised in a rabbit and further isolated from the serum via affinity chromatography. A detailed description of antigen production, immunization procedure and antibody purification process is given in sections 4.5.7 and 4.5.8.

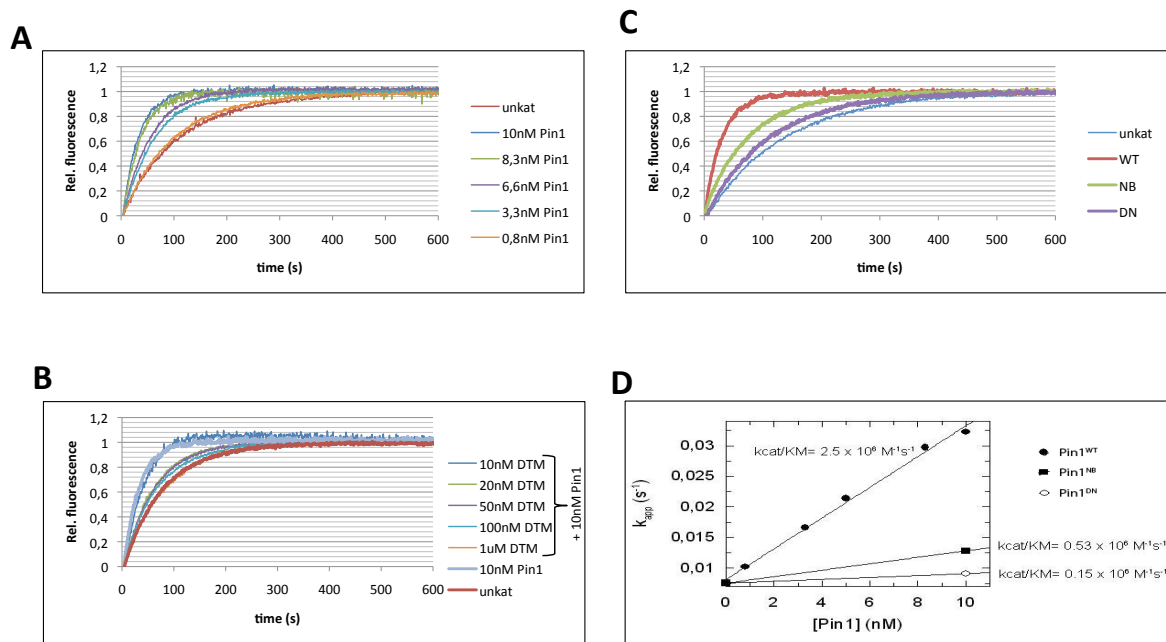
It was tested whether the xPin1-antibody recognizes its target in CSF extracts and whether it is suitable for immunodepletion. Meaningful results require complete depletion of Pin1 from the extract, as it cannot be ruled out that small residual amounts of Pin1 might be sufficient to retain functionality. Both the serum and the purified antibody detected a prominent band of approximately 18 kDa corresponding to the molecular weight of Pin1 in two independent CSF extracts. Furthermore, the majority of the anti-xPin1 antibody was depleted from the serum by the affinity purification as Pin1 was hardly detected by the flow-through of the affinity column. (Fig. 10A). Moreover, 10 µg of the purified antibody was sufficient to completely immunodeplete Pin1 from 50 µl of CSF extract in a single round (Fig. 10B). Given the high conservation of Pin1's primary structure between *Xenopus laevis* and *Homo sapiens* (93.3% similarity, 87.1% identity with 2.5% gaps), it was tested whether the antibody could also be used for detection of human Pin1 in Western blot (WB) and immunofluorescence (IF) approaches: Indeed, the antibody recognized a band at the expected mass of 18 kDa (Fig. 10C). No cross-reactive bands were detected in human cell lysates. Furthermore, it is further suitable for immunofluorescence microscopy (IF) of fixed human cells and for immunoprecipitation of endogenous Pin1 from human cell lysates (data not shown).

Next it was tested, whether bacterially expressed xPin1 was enzymatically active. For that purpose a peptide substrate was used which had a fluorophore and a quencher, coupled to the N- and C-termini, respectively. In its solvent, trifluoroacetic acid (TFA), the peptide is present in the *trans*- and *cis*-conformation with a ratio of 1:1. In the *cis*-conformation the fluorescence is suppressed by the close proximity of fluorophore and quencher. When adding this artificial substrate to an aqueous solution the equilibrium is shifted and the peptide gradually switches from the *cis*- into the *trans*-conformation

over the course of 5 to 10 minutes. This can be followed and quantified by measurement of the increase of fluorescence over time. Within this assay an accelerated increase in relative fluorescence is indicative of prolyl isomerase catalytic activity. Using this approach, the application of 3.3 nM of recombinant wild-type Pin1 was sufficient for inducing a rapid conformational switch of the peptide, as judged by the accelerated increase of the fluorescence. When using 10 nM recombinant wild-type Pin1 a maximum *in vitro* activity could be observed (Fig. 11A). Furthermore, the novel inhibitor DTM was tested for its capability to inhibit Pin1 activity *in vitro*. As shown in Fig. 11B, DTM strongly inhibited Pin1's enzymatic activity at a concentration of 20 nM and above as judged with by the decreased gradient of the fluorescence. Moreover, recombinant Pin1 variants, incapable of binding to substrates (Pin1<sup>NB</sup>) or inducing a conformational switch (Pin1<sup>DN</sup>), respectively, exhibited only a weak *in vitro* activity in comparison to wild-type Pin1 (Fig. 11C). The differences of the *in vitro* activities of the three recombinant Pin1 variants were further analyzed using a Lineweaver Burke diagram: In comparison to Pin1<sup>NB</sup> and to Pin1<sup>DN</sup>, data from the *in vitro* experiment using recombinant Pin1<sup>WT</sup> reproduces a significant gradient (Fig. 11D). Taken together, these results indicate that recombinant Pin1 and DTM can be used for experimental purposes: Bacterial expressed Pin1 exhibits high *in vitro* activity, while DTM acts as a strong Pin1 inhibitor. Moreover, bacterial expressed Pin1 variants (Pin1<sup>DN</sup> and Pin1<sup>NB</sup>) only exhibit a weak activity in comparison to wild-type Pin1.



**Fig. 10: Characterization of the xPin1 antibody:** **(A)** 0,3 μl of two independent CSF extracts were loaded on a SDS-PAGE followed by immunoblotting using the purified xPin1 antibody (concentration 0,4 μg/ml, left), serum before affinity purification of the antibody (1:1000 dilution, middle) and serum after affinity purification (1:1000, right). α-Tubulin served as a loading control. Asterisks (\*) mark non-specific bands due to cross-reactivity of unspecific IgG of the serum **(B)** The antibody was tested for its ability to deplete endogenous Pin1 from CSF extract: Extract was depleted using different amounts of xPin1 antibody coupled to 40 μl of magnetic protein G beads. Depletion was performed at 18°C for 45 min. After recovering the supernatant, the beads were washed several times with XB buffer. The samples were resolved on a SDS-PAGE gel followed by immunoblotting using the purified xPin1 antibody **(C)** The affinity purified xPin1 antibody was used to test whether it recognizes human Pin1 in different cell lines. Different cell lines from each 78cm<sup>2</sup> confluent culture dish area were directly lysed in 200 μl 1xSDS-sample buffer. For each sample 5 μl was loaded on a SDS-PAGE and analysed by immunoblotting using the purified x-Pin1 antibody at a concentration of 0,4 μg/ml. α-Tubulin served as a loading control.



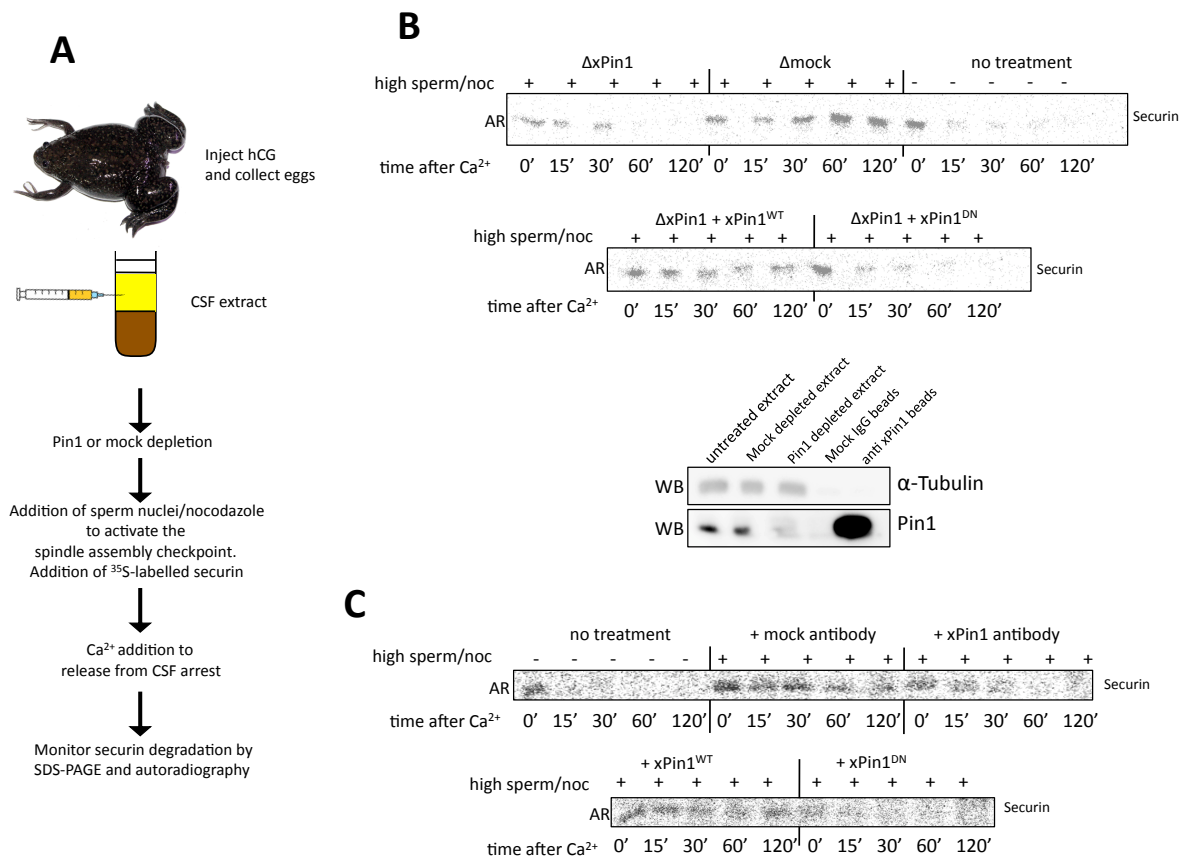
**Fig. 11: Enzymatic activity of wild-type- and mutant variants of Pin1 and validation of the novel Pin1 inhibitor DTM:** (A) Recombinant wild-type xPin1 from *E. coli* was tested in different concentrations for its *in vitro* activity in a peptide-based fluorescence assay. The relative fluorescence was measured and plotted against the time. (B) The same assay as in (A) was used to test the ability of DTM to inhibit recombinant xPin1 (10 nM). (C) Wild-type (WT) Pin1, dominant-negative (DN) or non-binding (NB) variants thereof were assayed as in (A) for comparison. Unkat = assay performed without addition of Pin1. (D) Lineweaver-Burke diagram generated with data from (A) and (C). *Experiments performed under guidance of Dr. Gabriel Zoldak.*

### 2.1.2. Pin1 is required for maintaining the SAC in *X. laevis* extracts

Having established its effective immunodepletion from *X. laevis* extracts, it was tested whether Pin1 is required for SAC functionality. Under conditions of an active SAC, securin levels should stay stable even when fertilization is mimicked by  $\text{Ca}^{2+}$  addition and the CSF-mediated inhibition of the APC/C is abolished. After one round of immunodepletion using anti-xPin1 or mock IgG, sperm nuclei (14,000/ $\mu\text{L}$ ), nocodazole and  $^{35}\text{S}$ -labeled human securin were added to the CSF extract followed by a short (5 min) incubation period at  $18^\circ\text{C}$ . Then,  $\text{Ca}^{2+}$  was added and securin stability assayed by taking samples at various time points followed by SDS-PAGE and autoradiography. As expected, securin stayed stable in a mock-depleted, SAC-arrested extract but was degraded when an untreated extract was released into interphase. Interestingly, Pin1-depleted SAC-activated extract also triggered securin degradation indicating SAC override (Fig. 12B). This override could be rescued when recombinant wild-type Pin1

(xPin<sup>WT</sup>) was added to depleted extract prior to Ca<sup>2+</sup>-activation. However, securin stayed unstable upon addition of a dominant-negative variant of Pin1 (xPin1<sup>DN</sup>) to Pin1-depleted extract, indicating that enzymatic activity of Pin1 is required for SAC signalling (Fig. 12C). Securin degradation could also be observed when xPin1 antibody or recombinant xPin1<sup>DN</sup> were simply added to SAC-activated extracts. Under these conditions securin was degraded within 60 minutes, whereas extracts supplemented with mock IgG or recombinant xPin1<sup>WT</sup> maintained a stable SAC-mediated arrest (Fig. 12C).

In summary, data obtained from these experiments strongly suggest that Pin1 might have an active role in maintaining the SAC in *X. laevis*. Strikingly, a Pin1-immunodepleted SAC-activated extract was unable to stabilize securin after release from the CSF-arrest, indicative of a checkpoint override. Furthermore, the above results suggests that not only the presence of Pin1 *per se*, but also enzymatic activity of Pin1 might be required for SAC maintenance, as addition of a catalytically inactive Pin1 variant (Pin1<sup>DN</sup>) to SAC-activated extract also triggered checkpoint override.



**Fig. 12: Pin1 is required for maintaining the SAC in *X. laevis*.** (A) Basic scheme of the experiment. (B) CSF extract was immunodepleted with anti-xPin1 or unspecific IgG (mock) and then supplemented with sperm nuclei (14,000/μl) and nocodazole (0,1 μg/20 μl extract) to trigger the SAC. Furthermore, recombinant xPin1<sup>WT</sup> or xPin1<sup>DN</sup> (10 μg/20 μl extract) was added to the xPin1-depleted extract. All extract samples were supplemented with  $^{35}\text{S}$ -labelled human securin and anaphase onset was triggered by addition of  $\text{Ca}^{2+}$  (two rounds of 0,6 mM). Samples were taken at the indicated time points and securin degradation was assessed by SDS-PAGE and autoradiography (AR). Depletion efficiency was analyzed by western blot (WB, lower two panels). (C) CSF extract was supplemented with high amounts of sperm nuclei and nocodazole as in (B) and with either mock IgG or xPin1 antibody (10 μg/20 μl extract), recombinant xPin1<sup>WT</sup> or xPin1<sup>DN</sup> (10 μg/20 μl extract). After addition of  $^{35}\text{S}$ -labelled securin and  $\text{Ca}^{2+}$ , samples were taken at the indicated times and separated on a SDS-PAGE followed by autoradiography to monitor securin degradation.

### 2.1.3. Pin1 has a role in maintaining the SAC in HCT116 and Hela cells.

Having found a putative role of Pin1 in SAC signalling during metaphase II in CSF extract, the question was raised whether Pin1 exhibits a similar function in mammalian cells during mitosis. *X. laevis* and human Pin1 share an amino acid identity of 87.1% and the sequences of their WW-domains (aa1-34) are identical for all but one amino acid (Fig. 13A). Consistently, proteins that are isomerised *in vitro* by human Pin1 usually are

also substrates of *X. laevis* Pin1 and *vice versa*. (Shen et al., 1998). This makes it likely that Pin1 exhibits similar functions in both systems. Therefore, it was tested whether inhibition of Pin1 with the novel chemical inhibitor DTM induces a checkpoint override in nocodazole treated, prometaphase arrested human cells. As the microtubule depolymerizing drug nocodazole prevents the formation of mitotic spindles, DNA segregation and cytokinesis cannot take place. Thus, nocodazole treated cells that are arrested in prometaphase and cells that enter anaphase due to a checkpoint override cannot be distinguished via staining of their DNA content. However, cells that enter anaphase dephosphorylate serine 10 of histon H3. Thus, cells entering anaphase due to a SAC override can be distinguished from those arrested in prometaphase in the presence of nocodazole by analyzing their content of unphosphorylated and phosphorylated histon H3 using flow cytometry (Gurley et al., 1974; Fig. 13B).

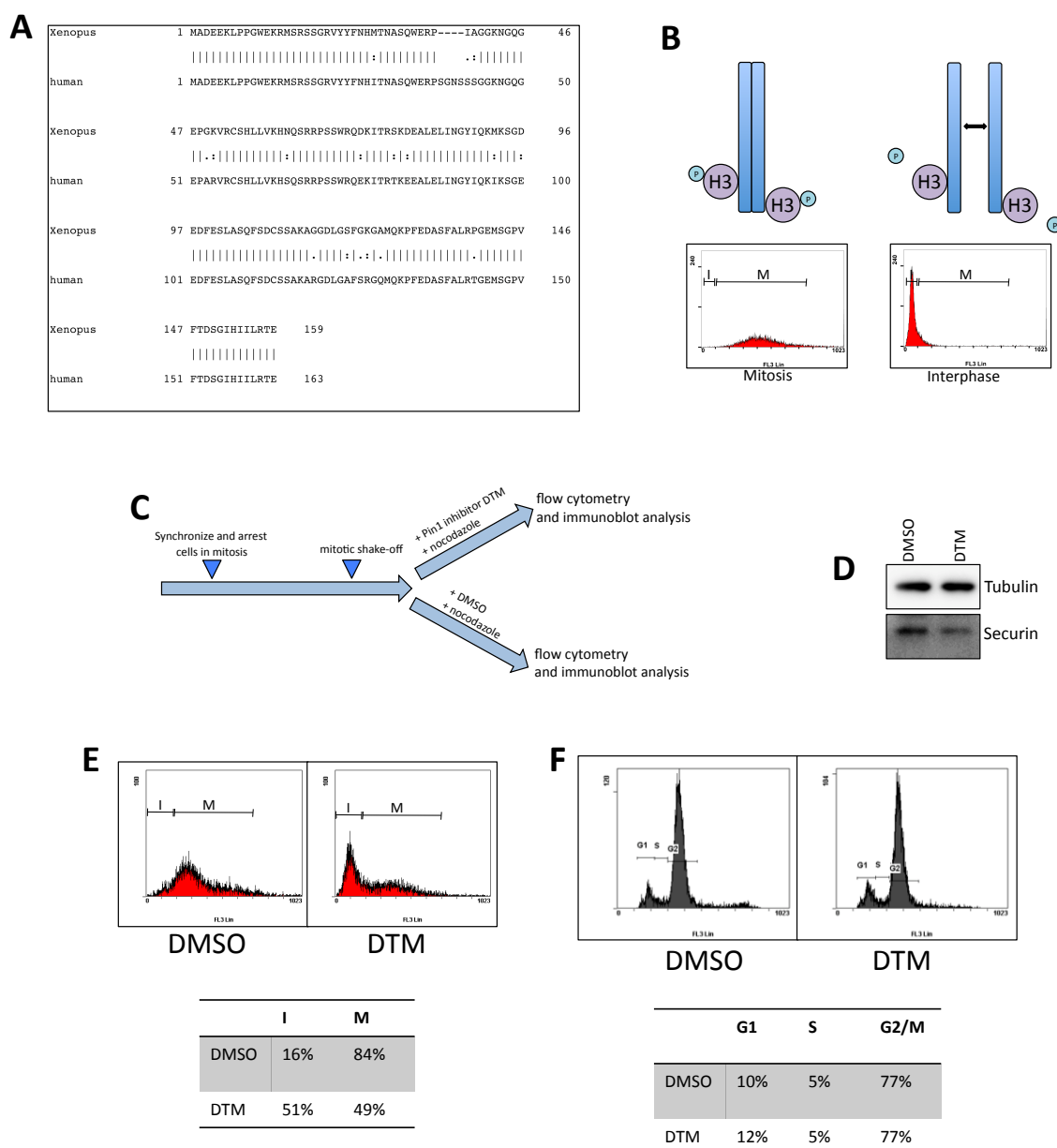
HCT116 cells were synchronised in mitosis using a double thymidine-nocodazole block and, after harvesting the cells by mitotic shake-off, they were either treated with DTM or DMSO, the corresponding solvent, for 7 h. Due to nocodazole treatment and disability to generate mitotic spindles, cells still exhibited a cell cycle distribution pattern with a G2/M peak, when stained with PI and analyzed by flow cytometry (Fig. 13F). To check for a potential checkpoint override, samples were immunoblotted against securin and the amounts of cells positive or negative for phosphorylated histon H3 were measured by flow cytometry. Interestingly, cells showed reduced securin levels upon Pin1 inhibition in contrast to DMSO-treated cells (Fig. 13D). Furthermore, in contrast to untreated cells which all seemed to contain phosphorylated histone H3, indicative of their arrest in prometaphase, most cells lost this phosphorylation upon Pin1 inhibition (Fig. 13E). The flow cytometry histogram of these cells contains a peak corresponding to unphosphorylated histone H3 and, hence indicating progression through mitosis.

A potential influence of Pin1 during SAC signalling could also be observed in Hela cells. These cells also exhibited a SAC override phenotype upon DTM treatment (Fig. 14). To strengthen the observed results, additional experiments were performed using epigallocatechin gallate (EGCG), a recently described, additional chemical inhibitor of Pin1 (Urusova et al., 2011). Thus, it was interesting, whether this specific Pin1 inhibitor induces similar effects as DTM. Upon eight-hour incubation with 100  $\mu$ M EGCG, no-

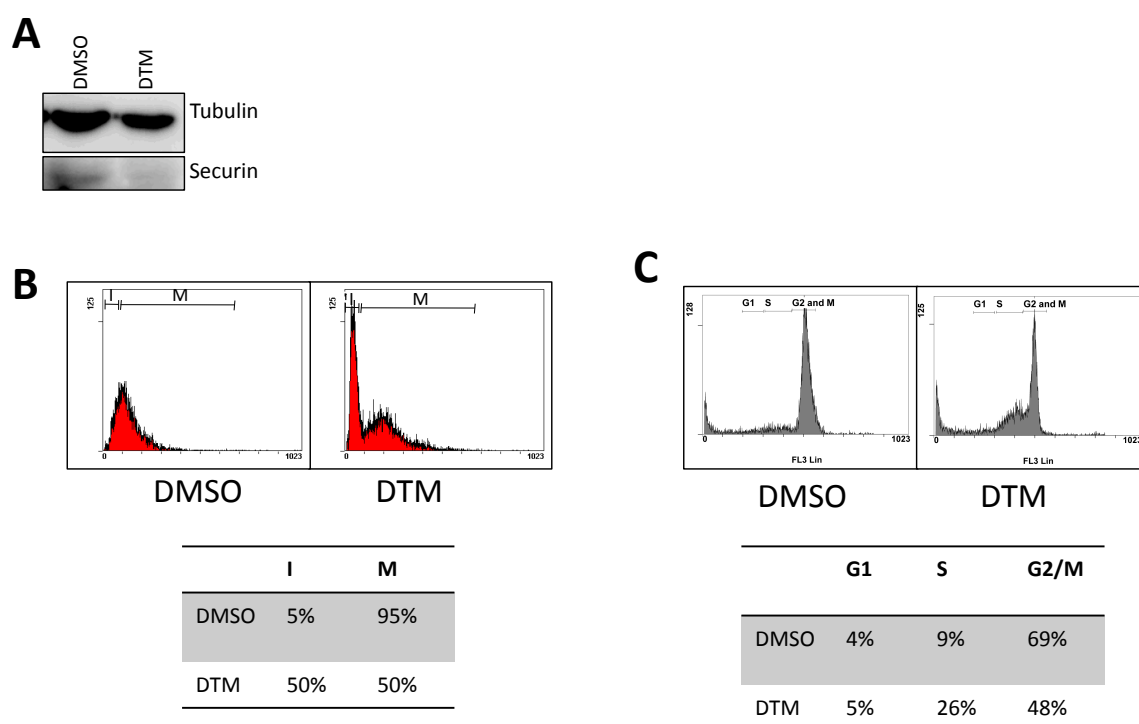


codazole arrested Hela cells lost almost all phosphorylation on serine 10 of histone H3 as determined by flow cytometry analysis (Fig. 15A). Similar observations could be made upon treatment of SAC-arrested HCT116 cells with this novel Pin1 inhibitor (data not shown). Consistent with an override of the SAC, mitotic Hela cells treated with either DTM or EGCG also showed a reduction in securin levels indicating checkpoint override (Fig. 15B). It was further checked, in what time frame the kinetics of a SAC override in response to Pin1 inhibition could be observed. To this end, SAC-arrested mitotic Hela cells were either treated with DTM or DMSO and samples were taken every hour and used for immunoblotting. Interestingly, strong effects on cyclin B1 and phosphorylated histone H3 levels couldn't be observed until 7 h after DTM treatment. However, after this time point, levels were greatly reduced. (Fig. 15C).

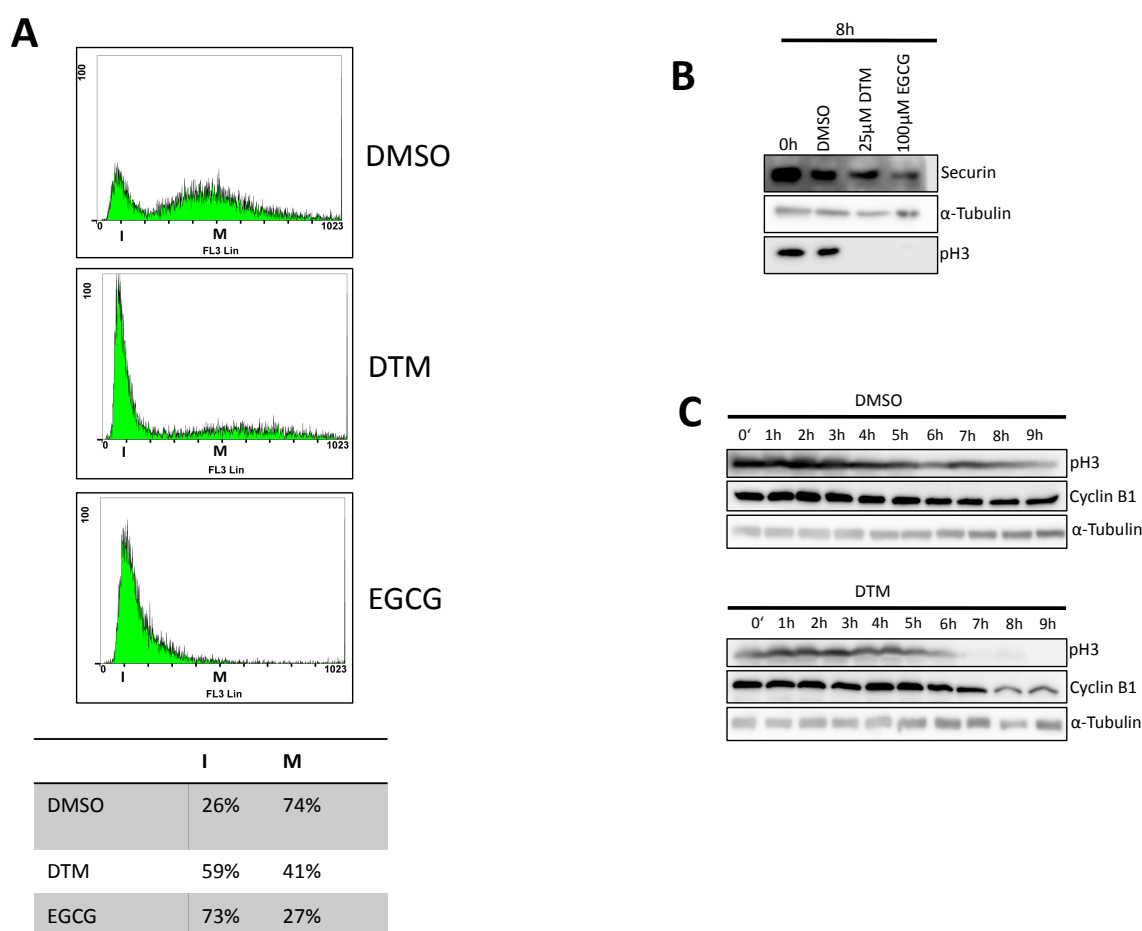
In summary, prometaphase arrested Hela or HCT116 cells were able to maintain their SAC for up to 7 h in the absence of Pin1 function/activity but then exited into interphase as judged by the loss of mitotic phosphorylation of histone H3 and degradation of the separase inhibitors cyclin B1 and securin. The slow kinetics of mitotic slippage suggests that Pin1 is crucial during a prolonged SAC mediated arrest.



**Fig. 13: Pin1 is required for maintaining the SAC in HCT116 cells:** (A) Sequence alignment of human and *X. laevis* Pin1. Identical residues, chemical similarities, and gaps are represented by vertical lines, two dots, and horizontal lines, respectively. (B) Cartoon showing distribution of phosphorylation of histone H3 in prometaphase arrested and interphase cells and its measurement by flow cytometry. (C) Basic scheme of the experiment: HCT116 cells were arrested in mitosis with a double thymidine-nocodazole block. Mitotic cells were harvested, transferred to nocodazole containing medium and treated with DMSO or 25  $\mu$ M DTM for 7 h. Samples were either used for an anti-securin and - $\alpha$ -tubulin immunoblot (D), measurement of distribution of phosphorylated histone H3 (E) or for analysis of cell cycle distribution as judged by PI staining (F). Diagrams of (D-E) show summarized data obtained from the flow cytometry histograms above. Missing numbers of the table in (E) refer to the sub-G1 population of the cells as an indicator of apoptosis. I=interphase cells, M=mitotic cells.



**Fig. 14: Pin1 is required for maintaining the SAC in Hela cells: (A-C)** Hela cells were arrested in mitosis with a double thymidine-nocodazole block and treated as in Fig. 13. Samples were either used for an anti-securin and  $\alpha$ -tubulin immunoblot (**A**), measurement of distribution of phosphorylated histone H3 (**B**) or for analysis of cell cycle distribution as judged by flow cytometry analysis of PI-stained cells (**C**). Tables of (B-C) summarize data obtained from the flow cytometry histograms above. I=interphase cells, M=mitotic cells. Missing numbers of the table in (C) refer to the sub-G1 population of the cells.



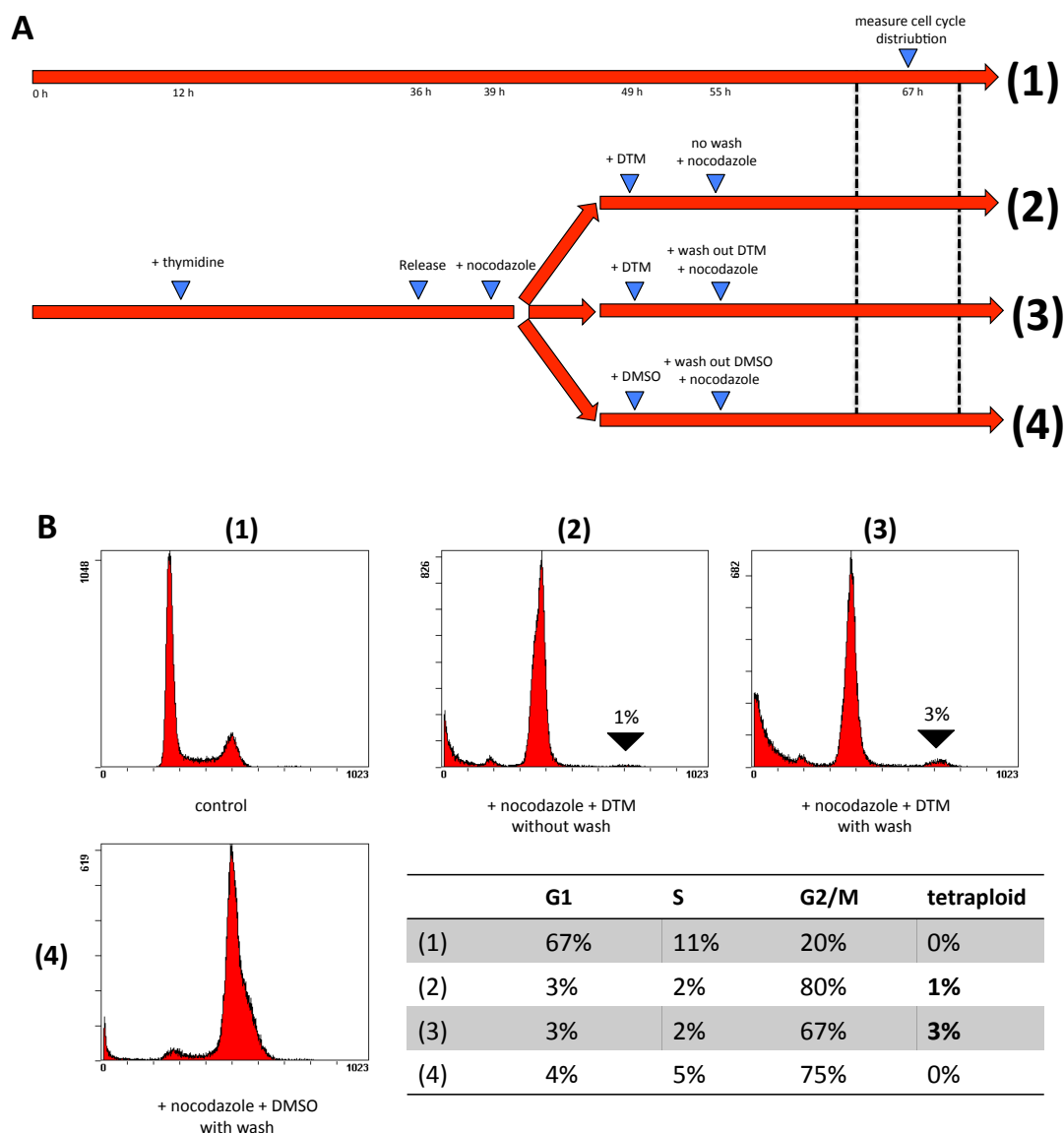
**Fig. 15: Pin1 activity is required for a robust, long-lasting SAC-mediated arrest: (A)** Experiment was performed as in Fig. 14B, but Pin1 inhibitor EGCG was used instead of DTM. The table summarizes data obtained from the flow cytometry histograms above. I = interphase, M = mitosis **(B)** HeLa cells were treated as in Fig. 14 but samples were used for SDS-PAGE and immunoblot analysis using antibodies against securin,  $\alpha$ -tubulin, and phosphorylated histone H3 (pH3) **(C)** HeLa cells were treated as in Fig. 14 but, beginning with the addition of DTM or DMSO, samples were taken every hour, separated by SDS-PAGE and analyzed by immunoblotting using antibodies against the indicated proteins.

#### 2.1.4. Pin1 inhibition in nocodazole arrested HCT116 cells partially induces tetraploidy

Having observed a potential SAC override phenotype in HCT116 and HeLa cells using two different Pin1 inhibitors, it was interesting to test whether the thesis of the involvement of Pin1 in SAC signalling could be supported by additional observations. Normally, SAC override causes premature sister chromatid segregation. However, in a nocodazole environment spindle microtubules do not form and sister chromatid segregation cannot take place even when the SAC is overruled and separase activated. More-

over, Pin1 inhibition during mitosis might lead to a late mitotic arrest, as Pin1 is needed for faithful cytokinesis (van der Horst and Khanna, 2009). To check for other consequences of Pin1 inhibition during SAC signalling, Pin1 was inhibited with DTM only after cells had entered mitosis synchronously. After six hours of incubation, DTM but not nocodazole was washed out. This step was made to ensure that cells leaving prometaphase due to Pin1 inhibition and potential checkpoint override do not fail to exit mitosis because of SAC-independent essential functions of Pin1 in cell cycle progression. After another incubation period of 12 hours, PI staining and flow cytometry were performed to determine cell cycle distribution. Interestingly, 3% of the cells in the DTM-treated sample were tetraploid indicating they had overridden the SAC in a nocodazole environment despite the disability to separate chromosomes and were partially able to initiate S-phase, although no faithful chromosome segregation could take place. In contrast, samples in which DMSO instead of DTM had been washed out, contained no or merely 1% tetraploid cells, respectively (Fig. 16B).

In summary, inhibition of Pin1 in prometaphase arrested HCT116 cells partially induces tetraploidy, indicative of a checkpoint override followed by cell cycle progression without previous chromosome segregation. Similar to the results of the previous experiments, these data also indicate an involvement of Pin1 in SAC maintenance.

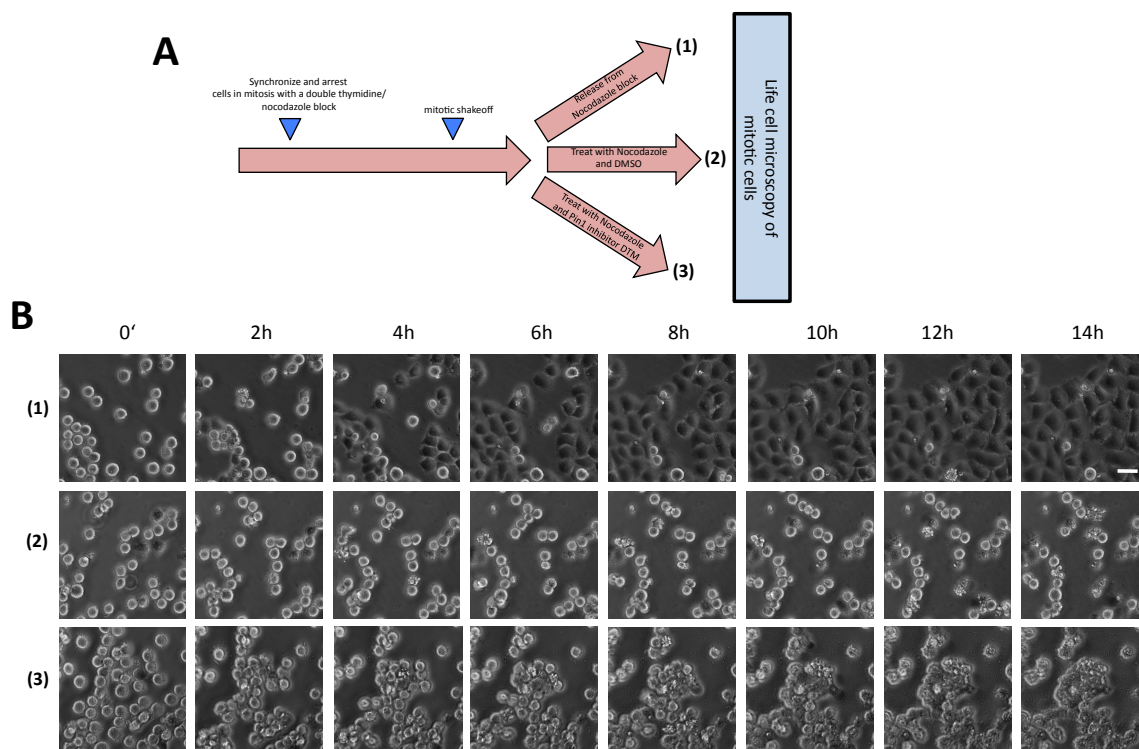


**Fig. 16: Pin1 inhibition during mitosis partially induces tetraploidy.** **(A)** Schematic overview of the experimental outline. **(B)** HTC116 cells were arrested in mitosis with a thymidine-nocodazole block. After mitotic shake off, cells were transferred to fresh medium containing nocodazole and 25  $\mu$ M DTM. After a 6 h incubation period, cells were harvested and washed twice for 5 min with 1xPBS containing nocodazole and DTM (2) or only nocodazole (3). Cells were further incubated in nocodazole-containing medium for another 12 h. Control cells were left untreated (1) or treated as in (3) using DMSO instead of DTM (4). Finally, cells were harvested, PI stained and analysed by flow cytometry. The table summarizes data obtained from the flow cytometry histograms. Missing numbers of the table refer to the sub-G1 populations of the cells. If present, cell populations with a 4n DNA content are marked in their position by arrowheads.

### 2.1.5. Inhibition of Pin1 forces prometaphase cells into a flattened morphology

It was further investigated whether changes in cell morphology could be observed upon Pin1 inhibition in SAC arrested prometaphase cells. To this end, mitotic Hela cells were isolated by releasing thymidine pre-synchronised cells into nocodazole (50 ng/ml). After a mitotic shake off and treatment with either DMSO or 50  $\mu$ M DTM in combination with nocodazole, life cell microscopy was performed and pictures were taken every 15 min. Interestingly, cells treated with DTM seemed to behave differently from those treated with DMSO. Whereas all DMSO-incubated cells showed a mitotic phenotype even after 14 hours and stayed rounded, Pin1 inhibition caused many cells to flatten and adhere to the substratum (Fig. 17). This interphase-like morphology was different from apoptosis as judged by the absence of apoptotic features like cell shrinkage or nuclear fragmentation.

In summary, the morphological changes of the cell shape upon prolonged inhibition of Pin1 in prometaphase arrested cells are consistent with the previous results and further substantiates the proposed SAC-supporting function of Pin1.



**Fig. 17: Pin1 inhibition forces cells to exit mitosis as judged by morphological changes: (A)** Schematic overview of the experiment. **(B)** Hela cells were arrested in mitosis with a double thymidine-

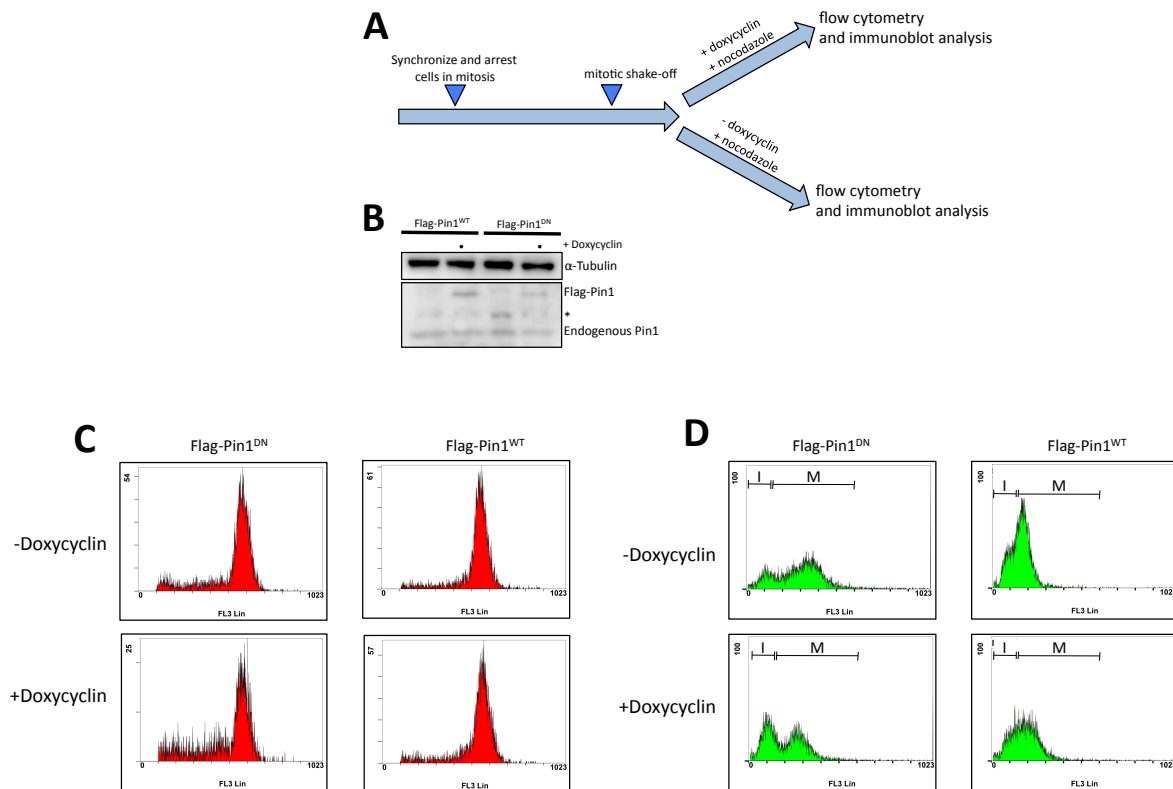
nocodazole block using low nocodazole concentrations (50 ng/ml). Upon mitotic shake off, they were either washed several times with 1xPBS and released into fresh medium (1), or transferred into nocodazole containing medium supplemented with either DMSO (2) or 50  $\mu$ M DTM (3). Life cell microscopy was performed and pictures were taken at the indicated times. Scale bar is 20  $\mu$ m.

#### **2.1.6. Inducible expression of Pin1<sup>DN</sup> abrogates a SAC-mediated mitotic arrest**

All previous cell culture experiments, which indicated the involvement of Pin1 in SAC maintenance, were performed using the specific chemical Pin1 inhibitors DTM or EGCG. However, it would be advantageous to check for a putative SAC failure using additional approaches. Earlier in this study, it could be shown that addition of a bacterially expressed dominant negative variant of xPin1 to SAC-activated extracts from *Xenopus* eggs induced checkpoint override (Fig. 12C). Thus, it was interesting to check, whether the corresponding human Pin1 variant could induce similar effects. To this end, stable transgenic HeLa cell lines were generated, which inducibly express Flag-tagged variants of either wildtype (Pin1<sup>WT</sup>) or dominant negative Pin1 (Pin1<sup>DN</sup>). The advantage of this system is that similar to Pin1 inhibition expression can be switched on only after the cells have already entered mitosis. Similar to previous experiments, the transgenic HeLa cells were pre-synchronized in early S-phase with thymidine and then arrested in mitosis with nocodazole. These mitotic cells were induced with doxycycline to express the transgene and finally harvested 8 h thereafter. Expression success was controlled by immunoblotting (Fig. 18B). Western blot analysis demonstrated, that the expression levels of exogenous Flag-tagged Pin1 variants are comparable to each other and to those of endogenous Pin1. The harvested cells were processed for flow cytometry analysis. Interestingly, cells expressing Pin1<sup>DN</sup> contained less serine 10-phosphorylated histone H3 and more unphosphorylated H3, indicating exit from mitosis by SAC override for part of the population. In contrast to Pin1<sup>DN</sup>, cells inducibly expressing Flag-tagged wild-type Pin1, didn't display any great difference as judged by flow cytometry (Fig. 18C). Due to nocodazole treatment, both cell lines still exhibited a cell cycle distribution pattern with a G2/M peak, when stained with PI (Fig. 18D).

In conclusion, data from this experiment indicates, together with previous results, that the enzymatic activity of Pin1 is required for maintenance of the SAC.





**Fig. 18: Overexpression of a dominant negative Pin1 variant causes HeLa cells to leave mitosis in the presence of spindle toxin. (A)** Schematic overview of the experimental outline: Stable transgenic HeLa cell lines that express Flag-tagged wildtype (Pin1<sup>WT</sup>) or dominant negative Pin1 (Pin1<sup>DN</sup>) were arrested in mitosis with a double thymidine-nocodazole block. After mitotic shake off, cells were harvested and transferred to fresh medium containing nocodazole and doxycycline (+ doxycycline) or the corresponding carrier, ethanol (- doxycycline). 8 h after induction, cells were harvested and used for expression analysis of endogenous Pin1 and the transgenes (Flag-Pin1) by immunoblotting (**B**), analysis of the cell cycle distribution via PI staining (**C**) or measurement of phosphorylated histone H3 distribution (**D**). I=interphase cells, M=mitotic cells. The asterisk (\*) in (A) marks a non-specific band due to cross-reactivity of the xPin1 antibody.

## 2.2. Pin1 interacts with checkpoint target Cdc20

Having elucidated a role of Pin1 in the SAC, the next step was to identify its potential targets. Phosphorylated histone H3 and securin/cyclin B1 levels stay constant for at least six hours in cells that lacked Pin1 function. Thus, Pin1 cannot be regarded as an essential component of the SAC but rather an additional factor that supports long-term mitotic arrests and is important for the robustness of the SAC.

Pin1 substrates to come into consideration were either checkpoint kinases, like Bub1 or aurora B, components of the MCC or subunits of the APC/C.

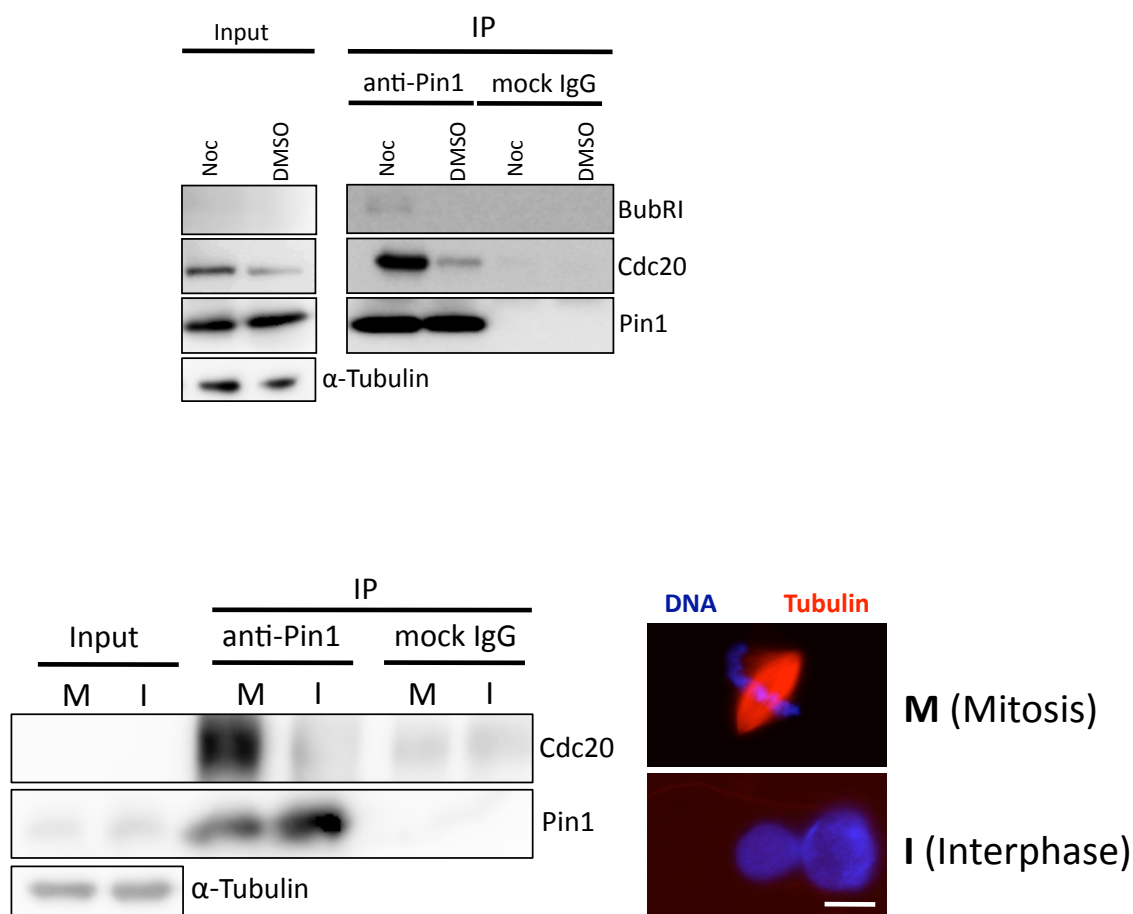
### **2.2.1. Pin1 interacts with the essential APC/C co-activator Cdc20 during mitosis**

Cdc20 is the key downstream target of the SAC. It contains eight S/P and T/P sites, seven of which were reported to be phosphorylated by Cdk1 *in vitro* (Yudkovsky et al., 2000). Furthermore, Cdc20 is specifically phosphorylated in mitosis *in vivo*, which makes it a candidate protein for interaction with Pin1.

It was checked whether Pin1 associates with Cdc20 in mitosis. The polyclonal *Xenopus* Pin1 antibody, which recognizes also human Pin1 (Fig. 10C), was used for co-IP experiments of endogenous Pin1 from either nocodazole-treated or unsynchronized Hek293T cells. Indeed, Cdc20 coprecipitated with Pin1 and the interaction was strongly enhanced in mitotically arrested cells. Furthermore, BubR1 could also be detected in the Pin1 immunoprecipitate from SAC-arrested cells, indicating that Pin1 binds to Cdc20 or the MCC (Fig. 19A).

Next it was checked whether this interaction is conserved between humans and amphibians. Clear indications of an involvement of Pin1 during the SAC were initially made in the *X. laevis* cell free system, making it likely that Cdc20-Pin1 association also occurs here. CSF extract was supplemented with either  $\text{Ca}^{2+}$  or the corresponding solvent, sperm dilution buffer, for 30 min to maintain the CSF arrest or to release the extract into interphase, respectively. Then, endogenous xPin1 was immunoprecipitated from these extracts using the polyclonal xPin1 antibody as bait. In this approach, a strong interaction between Pin1 and Cdc20 could be observed. Strikingly, only mitotic Cdc20 associated with Pin1 and not interphase Cdc20, indicating that mitosis-specific interaction is conserved between human and *X. laevis* (Fig. 19C).

The above results demonstrate that Pin1 associates with Cdc20 in mitosis. However, they leave unanswered, whether this interaction is direct or bridged by other MCC components like BubR1. It is also possible that the association is bridged via the APC/C as Cdc20 and the MCC bind to the APC/C *in vitro* and *in vivo* and an association between the APC/C subunit Cdc27 and Pin1 has been reported.



**Fig. 19: Pin1 binds to human and *X. laevis* Pin1 during mitosis.** **(A)** Hek293T cells were treated with either DMSO or nocodazole (250ng/ $\mu\text{l}$ ) for 14 h. Cells were then harvested and an anti-Pin1- or mock-IP from the respective cell lysates was performed. Samples were separated by SDS-PAGE and analyzed by immunoblotting using antibodies directed against BubR1, Cdc20, Pin1 and  $\alpha$ -tubulin. **(B)** 200  $\mu\text{l}$  CSF extract was supplemented with low amounts of sperm nuclei and rhodamine labeled tubulin and treated with either 0.6 mM  $\text{Ca}^{2+}$  or the corresponding solvent, sperm dilution buffer, for 30 min. Right panel: To test, whether the extracts were arrested in mitosis or released from the CSF-arrest into interphase, respectively, extract samples were taken and analyzed by immunofluorescence microscopy. DNA stained with Hoechst 33342 is shown in blue and rhodamine labeled tubulin in red. Scale bar is 5  $\mu\text{m}$ . Left panel: The extracts were then diluted with XB buffer in a ratio of 1:4. Following immunoprecipitation using antibodies directed against Pin1 or unspecific IgG (mock) for 2h at 18°C, the affinity matrices were washed 5 times with XB buffer. Proteins that remained bound to the matrices as well as input samples (0,2  $\mu\text{l}$  extract per lane) were separated by SDS-PAGE and used for immunoblotting of Cdc20, Pin1 and  $\alpha$ -tubulin.

### **2.2.2. The Cdc20-Pin1 interaction is direct and not bridged**

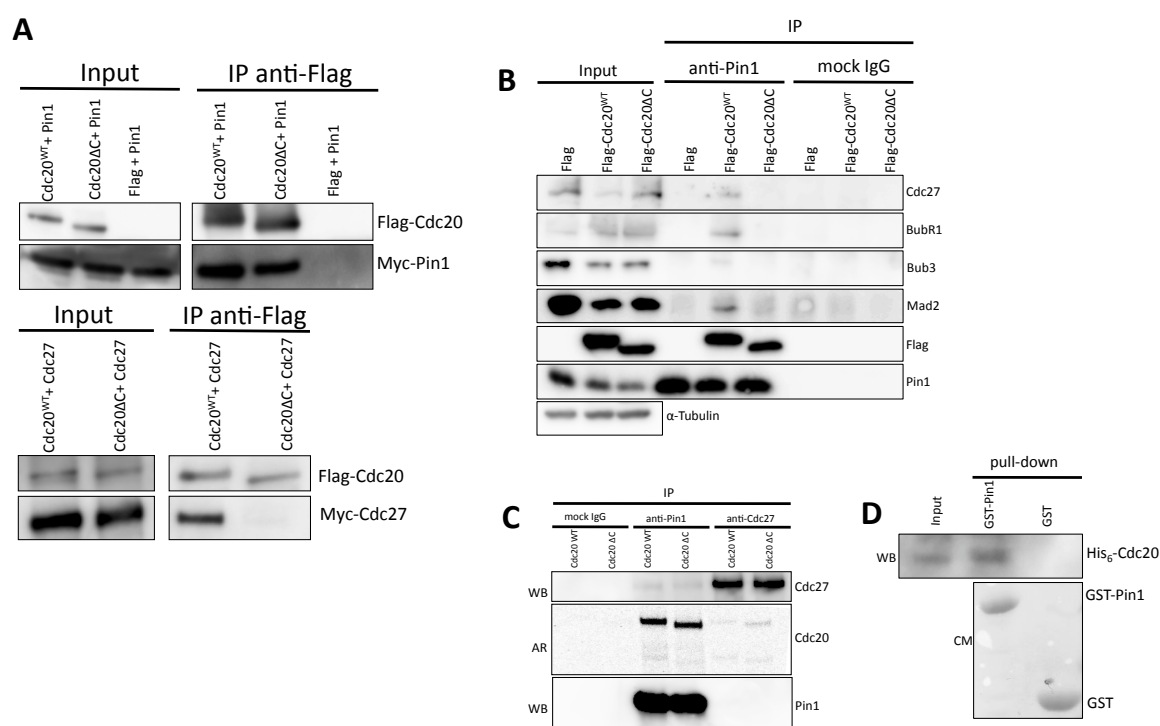
As Pin1 has many described mitotic substrates, it had to be excluded that Pin1-Cdc20 interaction is bridged by another component of the MCC like BubR1 or the APC/C. Concerning this matter, it has to be mentioned, that the APC/C subunit Cdc27 had been identified as a potential Pin1 target (Shen et al., 1998). Fortunately, the possibility of a bridging effect by the APC/C core can easily be addressed by using a C-terminally truncated variant of Cdc20 (Cdc20 $\Delta$ C), which lacks the last 29 amino acids and is unable to mediate interaction with Cdc27 or BubR1 (Amador et al., 2007).

Flag-tagged wildtype Cdc20 or Cdc20 $\Delta$ C were co-expressed with Cdc27 or Pin1 in Hek293T cells. After arresting the cells in mitosis by nocodazole treatment, immunoprecipitation from the corresponding cell lysates was performed using anti-Flag agarose. Indeed, co-IP of wildtype Cdc20 with Cdc27 could be observed. Furthermore, no interaction between Cdc27 and Cdc20 $\Delta$ C could be detected, however this C-terminally truncated Cdc20 variant still associated with Pin1, indicating that this association is not bridged by the APC/C. (Fig. 20A). Using another approach, Hek293T cells were transfected with plasmids coding for either full length Cdc20 or the C-terminally truncated Cdc20 variant (Cdc20 $\Delta$ C) and arrested in mitosis by nocodazole treatment. Cell lysates were prepared and endogenous Pin1 was immunoprecipitated. Upon IP of Pin1, all MCC components, Cdc27 and Bub1 kinase could be detected in the precipitate. The C-terminal truncated variant showed equally strong interaction with Pin1, however, the interesting observation could be made that almost no component of the MCC or the APC/C coprecipitated with Pin1 from the corresponding cell lysate (Fig. 20B).

It was further tested whether in CSF extracts the Pin1-Cdc20 interaction is also direct: Upon performing an endogenous IP from CSF extracts supplemented with <sup>35</sup>S-labeled wildtype Cdc20 or the C-terminally truncated variant of Cdc20, Pin1 showed strong association with both Cdc20 variants, whereas Cdc27 showed only a very weak interaction with Pin1 and Cdc20, indicating that Cdc20 doesn't even bind to the APC/C in *X. laevis* extract during the CSF arrest (Fig. 20C). This can likely be explained by sequestration of the APC/C by xErp1, which is mutually exclusive with a Cdc20-APC/C association.

Further indications that Cdc20 is a direct target of Pin1 were made in an *in vitro* pull-down assay using GST or GST-Pin1 loaded glutathione beads, respectively, and recombinant human Cdc20 (gift from E. Hörmannseider). Also in this approach, association between Pin1 and Cdc20 could be observed (Fig. 20D)

In summary, these above results strongly suggest, that Pin1 interacts with Cdc20 directly and this interaction is not bridged by another MCC component or the APC/C.



**Fig. 20: Interaction between Pin1 and Cdc20 is direct and not bridged:** **(A)** Upper Panel: Hek293T cells were transfected with plasmids coding for Flag tagged Cdc20 (WT = wild type, ΔC = C-terminal truncated) or the corresponding empty vector (Flag) and Myc tagged Pin1 followed by addition of 250 ng/ml nocodazole for 14 h to arrest the cells in mitosis. After an anti-Flag IP from the corresponding cell lysates, samples were separated by SDS-PAGE and immunoblotted using antibodies against the Flag- and Myc-epitopes. Lower Panel: Same approach as above but cells were transfected with plasmids coding for Myc-tagged Cdc27 and the Flag-tagged Cdc20 variants. **(B)** Flag-tagged Cdc20 or Cdc20ΔC were overexpressed in 293T cells followed by an mitotic arrest as in (A) and immunoprecipitation from the corresponding cell lysates using anti-Pin1 or unspecific IgG (mock) antibodies. Samples were separated by SDS-PAGE and immunoblotted using antibodies against the indicated proteins. **(C)** 4 μl of <sup>35</sup>S-labelled *X. laevis* Cdc20 variants from rabbit reticulocyte lysates were added to 150 μl CSF extracts. Following a 2 h incubation period at 18°C, IP from these extracts was performed using anti-xPin1, anti-Cdc27 or unspecific IgG (mock) antibodies. After intensive washing of the affinity matrices with XB buffer, proteins that remained bound were separated by SDS-PAGE. Pin1 and Cdc27 were detected by immunoblotting, while Cdc20 was visualized by autoradiography. **(D)** *In vitro* binding assay: His<sub>6</sub>-tagged Cdc20 purified from Sf9 cells was incubated

with GST- or GST-hPin1 loaded glutathione-beads in 1xPBS buffer for 1 h at RT. After intensive washing of the glutathione beads with 1xPBS/0,1% TritonX, proteins that remained bound as well as an input sample were separated by SDS-PAGE. GST- or GST-hPin1 were visualized by Coomassie stain (CM) and Cdc20 was detected by immunoblotting.

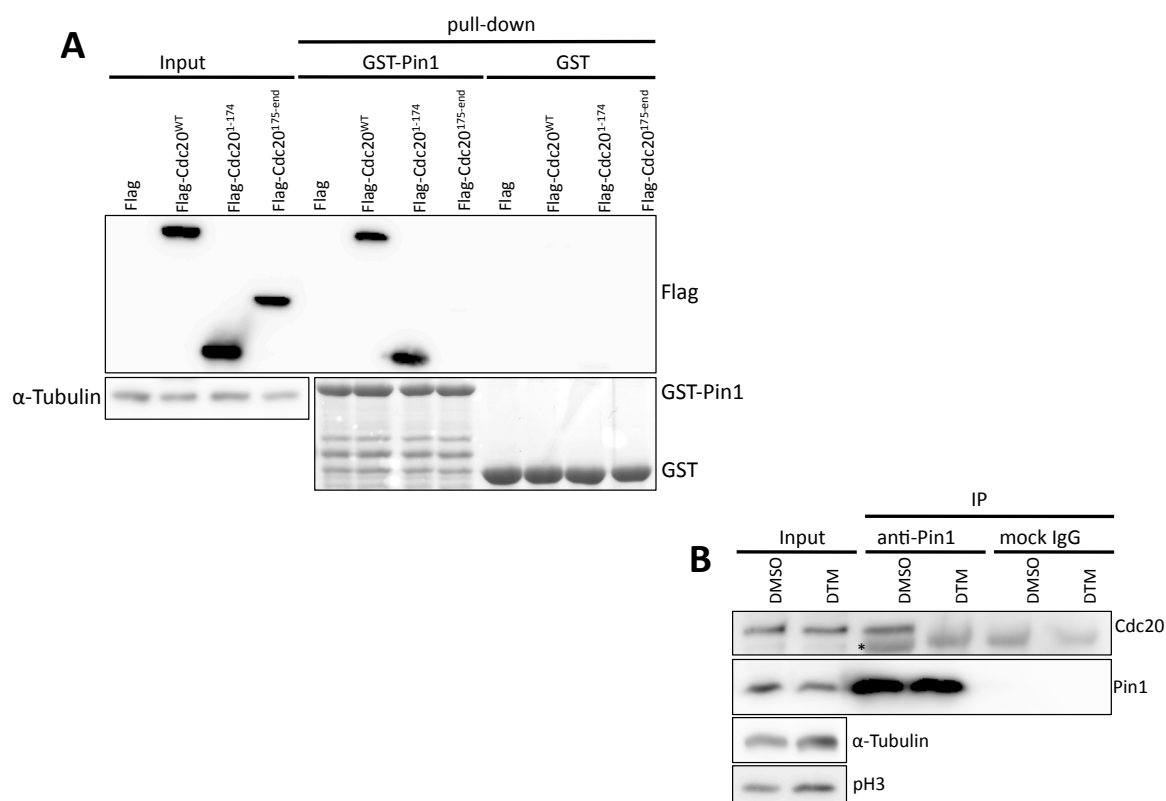
### **2.2.3. The N-terminus of Cdc20 mediates Pin1 interaction**

As described earlier, Cdc20 can roughly be divided into two domains. The C-terminal domain consists, amongst others, of short structural motifs, the WD-40 repeats. The N-terminal domain harbours most *in vivo* Cdk1 and Bub1 phosphorylation sites and mediates binding to Mad2 and at least three different APC/C subunits. Pin1 has a two-domain structure and consists of the N-terminal WW domain, required for proper substrate recognition and the C-terminal catalytically active PPlase domain. It would be appropriate to narrow down the Pin1 interaction region within Cdc20.

To this end, an *in vitro* pulldown assay was performed using GST or GST-Pin1 loaded glutathione beads from lysates of prometaphase arrested Hek293T cells transfected with plasmids coding for either full-length Cdc20 or N- or C-terminal fragments thereof. Using this approach, a strong binding between Pin1 and the N-terminal third of Cdc20 as well as the full length protein could be observed (Fig. 21A). No interaction between Pin1 and the C-terminal two thirds of Cdc20 was detectable.

It was further addressed whether binding of Pin1 to Cdc20 could be obstructed by its inhibitor DTM. To this end, mitotically arrested Hek293T cells were treated with DTM or its solvent DMSO for 3 h. Thereafter, an immunoprecipitation of the corresponding cell lysates was performed using anti-xPin1 and unspecific IgG (mock) antibodies. Indeed, Cdc20-Pin1 association was obstructed when adding DTM to mitotic cells (Fig. 21B), indicating that Pin1-Cdc20 binding requires Pin1's WW and PPlase domains and is not due to unspecific interaction of Cdc20 with Pin1.

Taken together these results indicate, that Pin1-Cdc20 interaction is mediated by the N-terminal third of Cdc20. This result is not unexpected as most of Cdc20's regulatory and binding sites lie within this domain. Furthermore the above data strongly suggests that Cdc20 is most likely a substrate of Pin1 as unspecific interaction between Cdc20 and Pin1 can be excluded.



**Fig. 21: Pin1 binds to the N-terminal third of Cdc20. (A)** 293T cells were transfected with plasmids coding for Flag-tagged full length Cdc20 (Cdc20<sup>WT</sup>), truncated N- or C-terminal variants thereof (Cdc20<sup>1-174</sup> or Cdc20<sup>175-end</sup>) or the corresponding empty vector (Flag) and arrested in mitosis using a double thymidine-nocodazole protocol. Cells were harvested and the corresponding cell lysates were incubated with GST- or GST-Pin1 loaded glutathione beads. After extensive washing of the glutathione beads, proteins that remained bound as well as input samples were separated by SDS-PAGE and immunoblotted using the indicated antibodies. GST- or GST-Pin1 were visualized by Coomassie stain. **Pin1-Cdc20 interaction is perturbed by Pin1 inhibitor DTM (B):** Nocodazole arrested 293T cells were treated with either 25  $\mu$ M DTM or its solvent DMSO for 3 h followed by an IP of endogenous Pin1 or a mock-IP from the corresponding cell lysates. Incubation and washing of the affinity matrices was performed in the presence of DTM or DMSO, respectively. Samples were separated by SDS-PAGE and analyzed by immunoblotting using antibodies against Cdc20, Pin1,  $\alpha$ -tubulin and serine 10-phosphorylated histone H3 (pH3). The asterisk (\*) corresponds to the IgG heavy chain.

#### 2.2.4. Pin1-Cdc20 interaction requires Cdk1-dependent phosphorylation

For most known Pin1 substrates a phosphorylation dependent binding was described. Thus, it was checked whether the Cdc20-Pin1 association exhibits a phosphorylation dependence as well. Indeed there are a few exceptions in which Pin1 can bind to some unphosphorylated proteins or proteins that do not contain S/P or T/P motifs. (Shen et al., 2005).

To test whether Pin1-Cdc20 association depends on phosphorylation, an *in vitro* kinase assay was performed. It was not possible to purify *E.coli* expressed full-length Cdc20 in a soluble form. Hence, it was decided to express and purify only the amino acids 1-144. The same N-terminal fragment of a Cdc20 variant, which lacked all S/P or T/P sites (Cdc20<sup>5A</sup>) served as a control. The proteins were expressed in fusion with a His<sub>6</sub>-SUMO3 tag and coupled to Ni<sup>2+</sup>-NTA agarose beads. The beads were then treated with recombinant Cdk1 and MAPK, two kinases with preference for S/P and T/P sites, in the presence of radioactively labeled [ $\gamma$ -<sup>33</sup>P]ATP. After incubation with recombinant full-length Pin1, the Ni<sup>2+</sup>-NTA agarose beads were washed several times and the proteins that remained bound were separated by SDS-PAGE and analyzed by immunoblotting, autoradiography and Coomassie stain. Interestingly, Pin1 was retained only on the wildtype Cdc20 column and importantly, much more so when the Cdc20 fragment had been phosphorylated (Fig. 22A). In contrast, Pin1 failed to interact with Cdc20<sup>5A</sup> even when the variant had been pre-incubated with kinases and ATP. This result strongly indicates that Pin1-Cdc20 interaction is phosphorylation dependent.

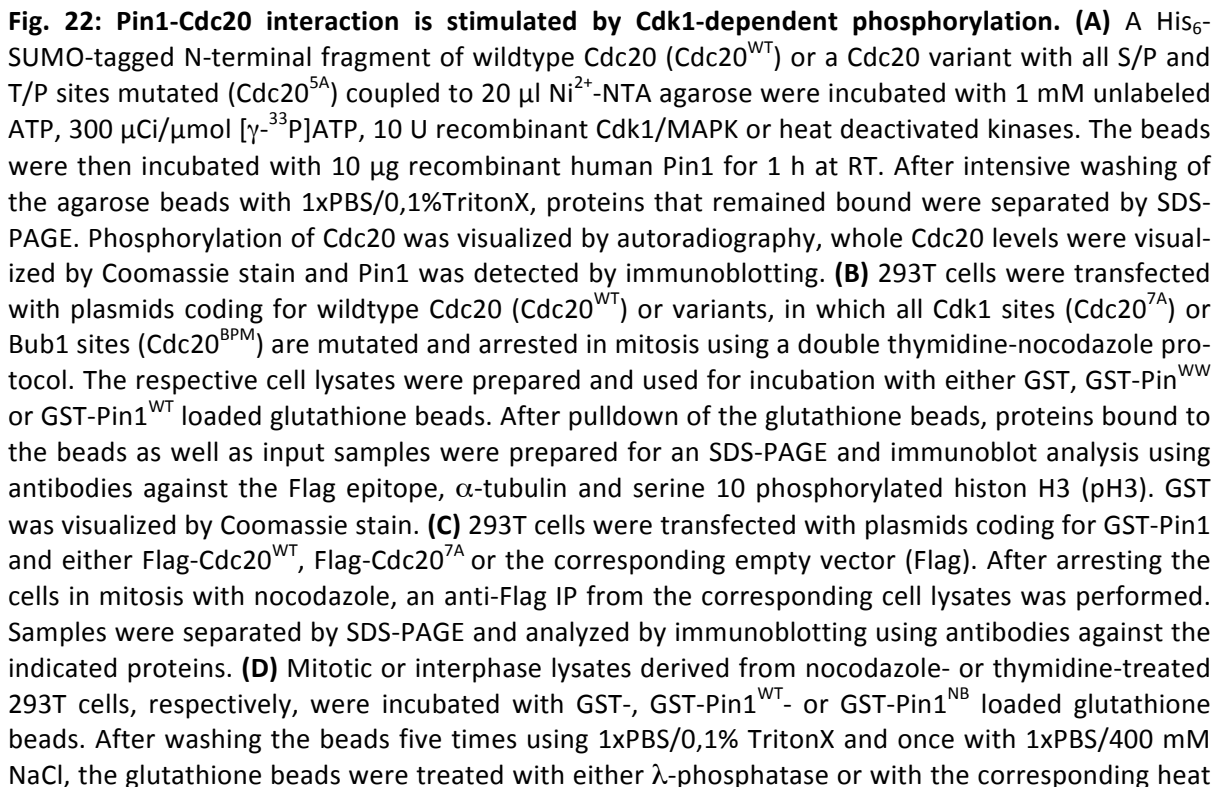
Using another approach, Hek293T cells were transfected with plasmids coding for wild-type Cdc20 (Cdc20<sup>WT</sup>) or a variant thereof in which all Cdk1 (Cdc20<sup>7A</sup>) or Bub1 (Cdc20<sup>BPM</sup>) phosphorylation sites were destroyed by mutation. After harvesting the cells, pulldown experiments from the corresponding cell lysates were performed using GST-, GST-Pin1, or GST-Pin1<sup>WW</sup>-loaded glutathione beads. Pin1<sup>WW</sup> only contains the WW-domain of Pin1 required for substrate recognition. Both the full length Pin1 and the Pin1 WW domain showed interaction with Cdc20<sup>WT</sup> but failed to associate with Cdc20<sup>7A</sup>. Furthermore Cdc20<sup>BPM</sup> showed equal ability to coprecipitate along with Pin1 indicating that Bub1-dependent phosphorylation of Cdc20 is dispensable for its interaction with Pin1 (Fig. 22B). In a similar approach GST-tagged Pin1 was co-expressed together with Cdc20<sup>WT</sup> or Cdc20<sup>7A</sup> in Hek293T cells, followed by an anti-Flag IP from the respective cell lysates. Here, only Cdc20<sup>WT</sup> but not Cdc20<sup>7A</sup> showed interaction with Pin1 (Fig. 22C).

In a final approach, recombinant wildtype Pin1 (Pin1<sup>WT</sup>) or a non-binding variant thereof (Pin1<sup>NB</sup>) were expressed in fusion with a GST tag and coupled to glutathione sepharose beads and incubated in lysates derived from mitotic or interphase Hek293T



cells. After intensive washing, the sepharose beads were then treated with  $\lambda$ -phosphatase or the corresponding heat inactivated enzyme, respectively. After another extensive washing procedure of the glutathione sepharose, the proteins that remained bound were analyzed by SDS-PAGE and immunoblotting. Strikingly, Cdc20 was retained only on the wildtype Pin1 column that was pre-incubated with the lysate derived from mitotic Hek293T cells. Furthermore, after incubation of these beads with  $\lambda$ -phosphatase, Pin1 lost affinity to Cdc20 and levels were reduced to background (Fig. 22D).

Thus, these data strongly suggest that Pin1-Cdc20 indeed depends on Cdk1 activity and specifically occurs during mitosis. These results come at no surprise, as Pin1 has a preference for mitotic substrates and binding to its substrates during mitosis mostly depends on Cdk1 activity.



deactivated enzyme. The glutathione beads were then again washed and proteins that remained bound as well as input samples were separated by SDS-PAGE and immunoblotted using antibodies against Cdc20,  $\alpha$ -tubulin and phosphorylated histone H3 (pH3); GST-, GST-Pin1<sup>WT</sup>- or GST-Pin1<sup>NB</sup> were visualized with Coomassie stain. (WT = wildtype, NB = non binding)

### 2.3. Pin1 influences Cdc20 distribution

#### 2.3.1. Pin1 influences distribution of Cdc20 during SAC signalling

Motivated by the identification of the novel Cdc20-Pin1 interaction, the molecular consequences of this interaction were investigated. As shown earlier, Pin1 only associates with the N-terminal third of Cdc20 in mitotically arrested Hek293T cells. Thus, it would be comprehensive that Pin1 influences activity of functional sites, situated within this region. At least three APC/C binding sites, the Mad2 binding domain as well as destruction boxes are located in the N-terminal third of Cdc20. Hence, it is possible that Pin1 influences association of Cdc20 with either Mad2 or the APC/C during mitosis.

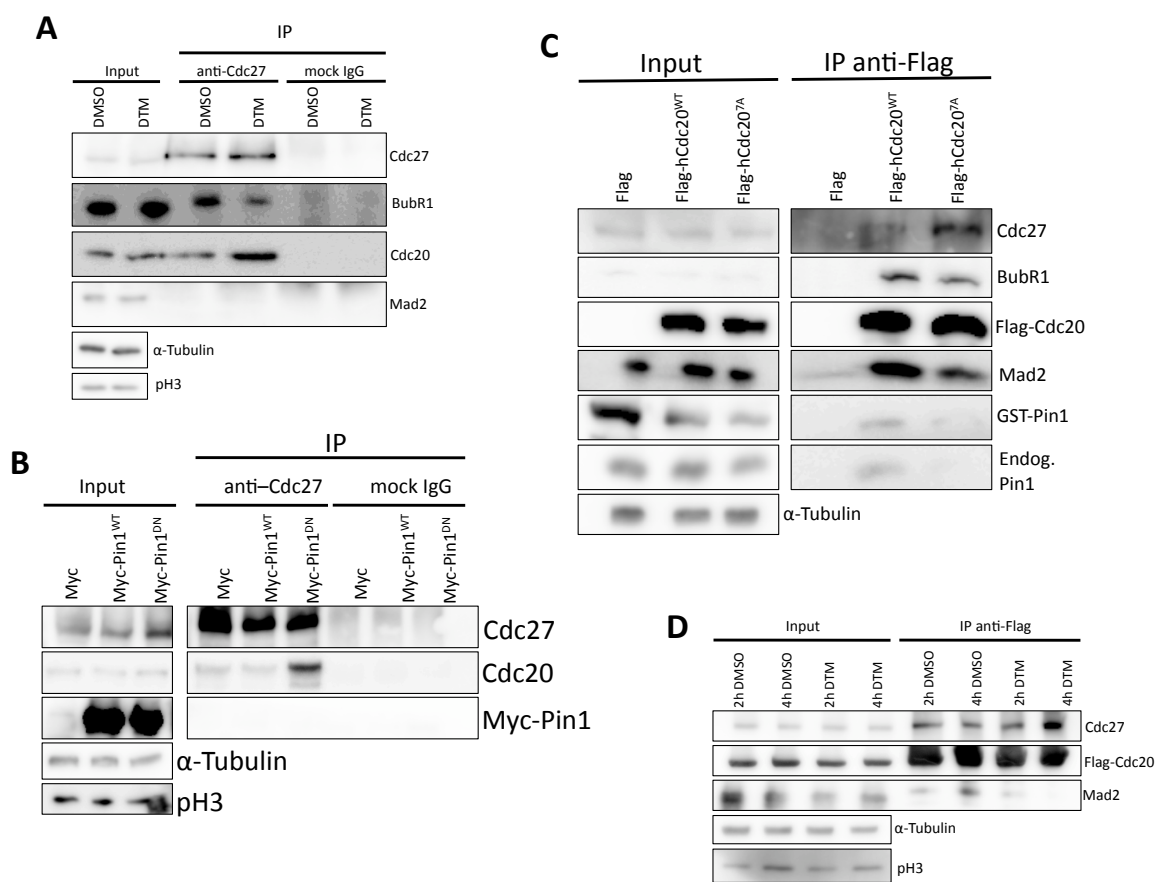
To address this issue, Hek293T cells were transfected with plasmids coding for wild-type Pin1 or a dominant negative variant thereof and arrested in prometaphase with a double thymidin/nocodazole block. Using a similar approach, Pin1 inhibitor DTM or the corresponding solvent (DMSO) were added to mitotically arrested Hek293T cells for 6 h. After harvesting the cells, immunoprecipitation of endogenous Cdc27 from the respective cell lysates was performed. Interestingly, increased association of Cdc20 with Cdc27 could be observed in cells treated with Pin1 inhibitor DTM compared to DMSO-treated cells. Furthermore, upon Pin1 inhibition also the amount of BubR1 bound to Cdc27 was strongly reduced, indicating that APC/C bound Cdc20 was not MCC associated (Fig. 23A). Similar to the above result, the Cdc20/Cdc27 interaction was also enhanced in mitotically arrested Hek293T cells expressing the dominant negative Pin1 variant compared to wild type Pin1 (Fig. 23B), indicating that the catalytic activity of Pin1 is required for proper Cdc20 distribution during SAC signalling.

In a third approach Hek293T were transfected with plasmids coding for Flag tagged wildtype Cdc20 (Cdc20<sup>WT</sup>) or a variant thereof lacking all Cdk1 phosphorylation sites (Cdc20<sup>7A</sup>) and GST-tagged Pin1 followed by a double thymidine-nocodazole block to arrest the cells in prometaphase. After performing an IP from the corresponding cell ly-

sates using anti-Flag agarose, the proteins bound to the affinity matrices as well as input samples were separated by SDS-PAGE and analyzed by immunoblotting. Interestingly, increased association of Cdc20<sup>7A</sup> with Cdc27 and simultaneously decreased association with Mad2 could be observed compared to wild type Cdc20. (Fig. 23C). Furthermore, expressed GST-tagged Pin1 and the endogenous protein failed to associate with Cdc20<sup>7A</sup>.

In a final approach, Hek293T cells, which were subsequently synchronized using a double thymidine-nocodazole protocol, were transfected with a plasmid coding for wildtype Cdc20. The prometaphase arrested cells were further treated with Pin1 inhibitor DTM or DMSO for 2 or 4 h. Cells were then harvested, lysed and subjected to an IP using anti-Flag agarose. Finally Flag-Cdc20 and associated proteins were analyzed by immunoblotting. Similar to the above results, Pin1 inhibition seemed to enhance binding of Cdc20 to the APC/C and simultaneously decreased its ability to bind to Mad2 (Fig. 23D).

In conclusion these data suggests that Pin1 influences the distribution of Cdc20 during mitosis. However, the above data does not clarify whether Pin1 mediates association with MCC or prevents premature association with the APC/C during a prolonged prometaphase arrest.



**Fig. 23: Pin1 influences distribution of Cdc20 during mitosis:** **(A)** Hek293T cells were arrested in mitosis using a double thymidine-nocodazole protocol followed by addition of DMSO or 50  $\mu$ M DTM for 6 h. After preparing the respective cell lysates, IP was performed using anti-Pin1 or unspecific IgG (mock) antibodies. Samples were separated by SDS-PAGE and immunoblotted using antibodies against the indicated proteins. **(B)** Hek293T cells were transfected with plasmids coding for Flag-tagged Cdc20<sup>WT</sup>, a variant in which all Cdk1-phosphorylation sites are mutated (Cdc20<sup>7A</sup>) or the corresponding empty vector (Flag) as well as GST-Pin1 and arrested in mitosis using a double thymidine-nocodazole block. An IP from the corresponding cell lysates was performed using anti-Flag agarose. Samples were analyzed by SDS-PAGE and immunoblotted using antibodies against the indicated proteins. **(C)** Hek293T cells were treated as in (A) but cells were transfected with plasmids coding for Myc-tagged Pin1<sup>WT</sup>, a dominant negative variant (Pin<sup>DN</sup>) or the respective empty vector (Myc) instead of DTM/DMSO treatment. An IP from the corresponding cell lysates was performed using anti-Cdc27 or unspecific IgG (mock) antibodies. Immunoblot analysis was performed using antibodies against the indicated proteins. **(D)** Hek293T cells were transfected with a plasmid coding for Flag-tagged wild type Cdc20 and arrested in prometaphase using a double thymidine-nocodazole protocol. Thereafter, DTM or the corresponding solvent DMSO were added to the cells for 2 or 4 h. After harvesting the cells, an IP from the respective cell lysates was performed using anti-Flag agarose. Samples were separated by SDS-PAGE and analyzed by immunoblotting using the indicated proteins

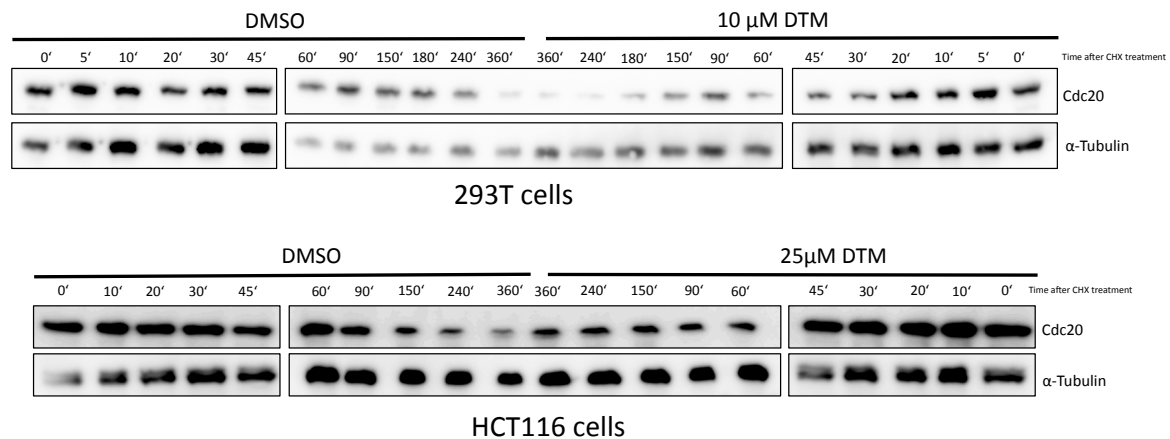
### **2.3.2. *Pin1 does not influence stability of Cdc20 during SAC signalling***

In most cases ubiquitylation of proteins triggers their proteasomal degradation. In this context, Pines and co-workers claim that constant degradation and re-synthesis of the APC/C co-activator Cdc20 is essential for prolonged checkpoint activity, possibly because exceeding amounts of Cdc20 are degraded. Consequently, premature activation of the APC/C is prevented (Nilsson et al., 2008). Previous results of this study indicate that Pin1 is required for the maintenance of the SAC. Furthermore Pin1 has been shown to influence the degree of ubiquitylation of a multitude of substrates. Thus, it is likely that Pin1 also influences the ubiquitylation levels of Cdc20 during mitosis.

To clarify this issue, different cell lines were synchronized in prometaphase using a double thymidine-nocodazole block. After mitotic shake-off, cells were treated with 10  $\mu$ M of the translation inhibitor cycloheximide (CHX) to prevent synthesis of Cdc20. Furthermore, DMSO or the Pin1 inhibitor DTM were added to the cells, which were harvested at different time points. The corresponding cell lysates were subjected to immunoblot analysis. Cdc20 levels were gradually reduced in Hek293T and HCT116 cells. However, in all cell lines tested, no significant impact of Pin1 on Cdc20 degradation was visible. Neither in Hek293T, nor in HCT116, it seemed that inhibition of Pin1 altered Cdc20 degradation dynamics (Fig. 24). Similarly, Pin1 activity had no impact on Cdc20 levels in Hela and U2OS cells (data not shown).

Using different approaches, it was tested whether Pin1 influences the degree of ubiquitylation of Cdc20 during a prolonged mitotic arrest. However, due to experimental difficulties, I was unable to gain meaningful results, but the obtained data rather argues against a strong impact of Pin1 on Cdc20 ubiquitylation (data not shown).

In summary, Pin1-Cdc20 interaction does not seem to influence the ubiquitylation and degradation of Cdc20 during a prolonged prometaphase arrest. Neither are there indications that Pin1 alters the degree of Cdc20 ubiquitylation nor does it seem that Pin1 influences Cdc20 stability in general.



**Fig. 24: Pin1 does not influence Cdc20 stability during a prolonged SAC:** 293T or HCT116 cells were arrested in mitosis with a double thymidine-nocodazole block followed by addition of 10 μg/ml CHX plus DMSO or Pin1-inhibitor DTM. Cells were harvested at the indicated time points and subjected to an immunoblot analysis using antibodies against Cdc20 and α-tubulin.

### 2.3.3. Inhibition of Pin1 impairs localization of Cdc20 to kinetochores

Several reports indicate kinetochore localization of Cdc20 during mitosis: This localization is established during prophase and abolished upon anaphase onset (Kallio et al., 2002). Furthermore localization at the kinetochores has been described for all MCC components (Vigneron et al., 2004). However, it is not clear yet, whether Cdc20 localization to kinetochores is a prerequisite for proper SAC functionality.

To clarify whether Pin1 influences Cdc20 localization, Pin1 was immunodepleted from CSF extracts which was further supplemented with low amounts of sperm nuclei. Subsequently, a chromatin re-isolation was performed. The chromatin was fixed on coverslips and used for immunofluorescence staining of Cdc20 and centromeres. Strikingly, Cdc20 signals were visibly reduced by Pin1-immunodepletion (Fig. 25B). However, using this approach it was not possible to clarify whether Pin1 directly influences Cdc20 localization to kinetochores, as most Cdc20 was co-depleted from the extract upon Pin1-immunodepletion (Fig. 25C). Hence, it was decided to address the issue of a possible impact of Pin1 on Cdc20 localization using mammalian cell culture.

To this end, Hela cells were depleted of Pin1 by RNAi and subsequently arrested in mitosis. After mitotic shake-off, cells were fixed and prepared for immunofluorescence analysis using antibodies against Cdc20 and the CREST human autoserum as a marker for centromeres. Interestingly, upon Pin1 depletion, Cdc20 levels at the kinetochore were

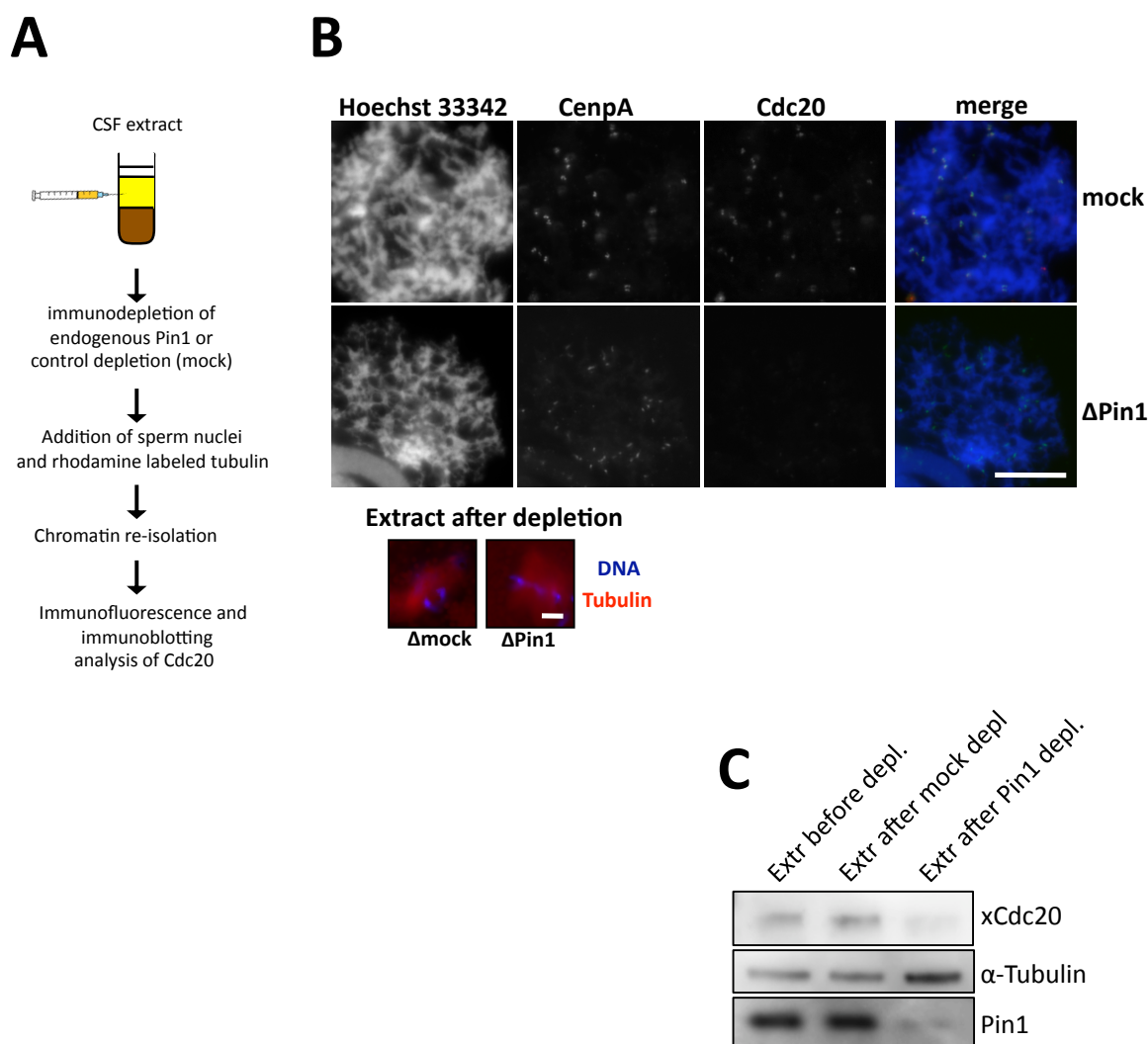
slightly reduced compared to mock (GL2) treatment (Fig. 26). Similar effects could be observed in mitotically arrested HeLa cells treated with Pin1 inhibitor DTM (data not shown). However, also in these approaches it cannot be excluded that treatment of the cells with RNAi or inhibition of Pin1 by DTM induces side effects such as co-depletion of Cdc20. It is furthermore possible that reduced Cdc20 levels at the kinetochores during mitosis are an indirect effect of Pin1 depletion.

To address the issue whether Pin1 directly influences Cdc20 localization, stable transgenic cell lines were generated inducibly expressing wildtype Cdc20 (Cdc20<sup>WT</sup>) or a variant thereof lacking all Cdk1 phosphorylation sites (Cdc20<sup>7A</sup>). In a previous experiment, this variant failed to associate with Pin1 (Fig. 22B). After a mitotic shake-off, cells were prepared for immunofluorescence analysis using antibodies against Cdc20 and the kinetochore marker Hec1. Using this approach, localization of both wildtype Cdc20 and Cdc20<sup>7A</sup> to the kinetochores was observed, indicating that Pin1 does not directly influence Cdc20 distribution to the kinetochores (Fig. 27A).

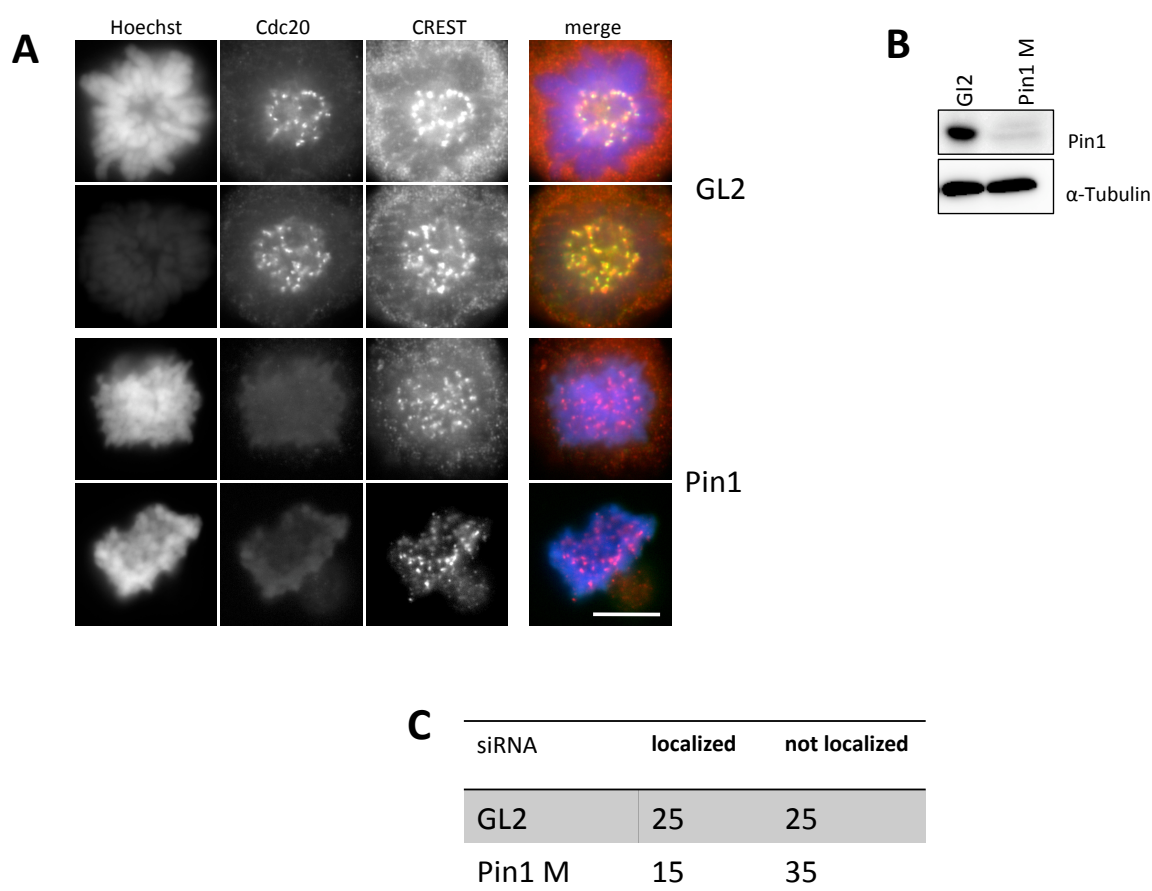
Hence, the question was raised, whether there are other mechanisms that direct Cdc20 to the kinetochores as Pin1 didn't seem to directly contribute to Cdc20 localization. To this end, HeLa cells were transfected with plasmids coding for different Cdc20 variants shown in Fig. 27B and arrested in mitosis with nocodazole. Thereafter, immunofluorescence analysis of the corresponding cells was performed and localization of Cdc20 to the kinetochores was analyzed. Cdk1- and Bub1-phosphorylation deficient Cdc20 variants still showed association with kinetochores, indicating that phosphorylation is not a prerequisite for proper Cdc20 localization. (Fig. 27C-E). However, a C-terminally truncated variant failed to localize to kinetochores, whereas a Cdc20 variant lacking the last two amino acids (unable to mediate association with Cdc27 or MCC component BubR1), still showed localization.

In conclusion, Pin1 does not seem to directly influence Cdc20 localization to kinetochores during mitosis. The localization of Cdc20 to kinetochores is neither controlled by phosphorylation nor by cis-trans isomerization. Instead it is regulated by an unknown mechanism, which requires the C-terminal end of Cdc20.

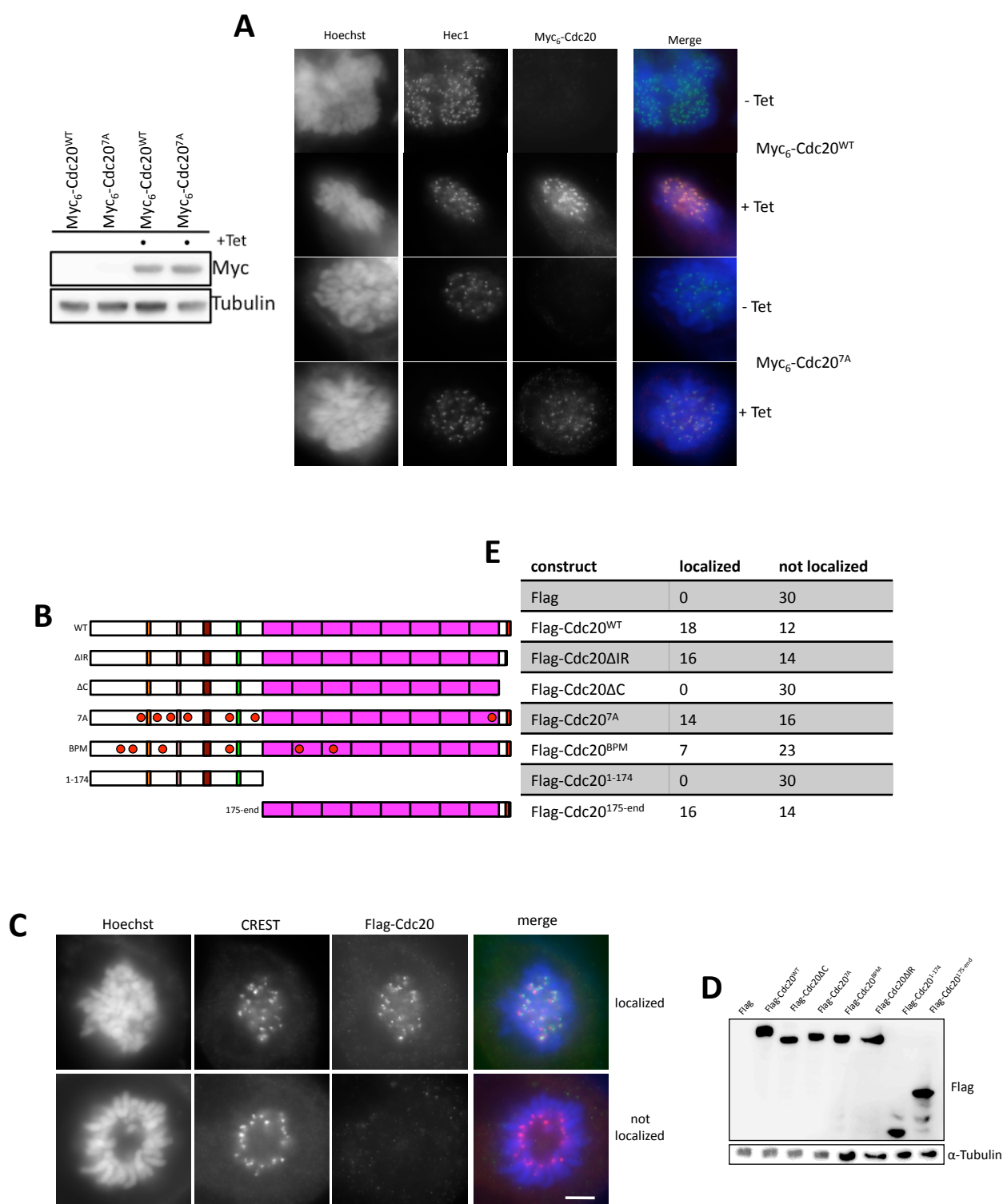




**Fig. 25: Pin1 depletion from CSF extract reduces the amount of total and kinetochore-associated Cdc20:** **(A)** Experimental outline of the experiment. In brief, CSF extract was immunodepleted with anti-xPin1 antibodies or unspecific IgG (mock) and then supplemented with low amounts of sperm nuclei and rhodamine labeled tubulin. After an incubation period of 30 min at 18°C, the sperm nuclei were re-isolated and analyzed by immunofluorescence microscopy **(B)** Upper panels: Representative IF pictures of re-isolated chromatin from mock or Pin1 depleted CSF extract. Chromatin was stained using antibodies against the centromere marker xCenpA (red) and Cdc20 (green). DNA was counterstained with Hoechst 33342. Scale bar is 20  $\mu$ m. Lower panels: Representative IF pictures of extract samples taken after the depletion process. DNA was counterstained with Hoechst 33342. Rhodamine labeled tubulin is shown in red. Scale bar is 20  $\mu$ m. **(C)** Extract samples taken after immunodepletion were subjected to an immunoblot analysis using antibodies against xCdc20,  $\alpha$ -tubulin and xPin1.



**Fig. 26: Pin1 depletion reduces the amount of kinetochore-bound Cdc20 in HeLa cells. (A-C)** HeLa cells were transfected with siRNAs directed against Pin1 mRNA (Pin1 M) or mock transfected (GL2) for 48 h. Following treatment with nocodazole for 14 h, mitotic cells were harvested by shake-off. Cells were then either spun on coverslips, fixed and stained for immunofluorescence microscopy or prepared for immunoblot analysis. **(A)** Immunofluorescence pictures of the corresponding cells stained with antibodies against Cdc20 (green) and CREST as a marker for centromeres (red). DNA was stained with Hoechst 33342. Scale bar is 10  $\mu$ m. **(B)** Lysates of mitotic cells were analyzed by SDS-PAGE and immunoblotting to assay efficiency of Pin1 depletion by RNAi. Mitotic cells (50 each) with kinetochoric (localized) and no detectable Cdc20 signal (not localized) were counted. Numbers are presented in a table **(C)**.

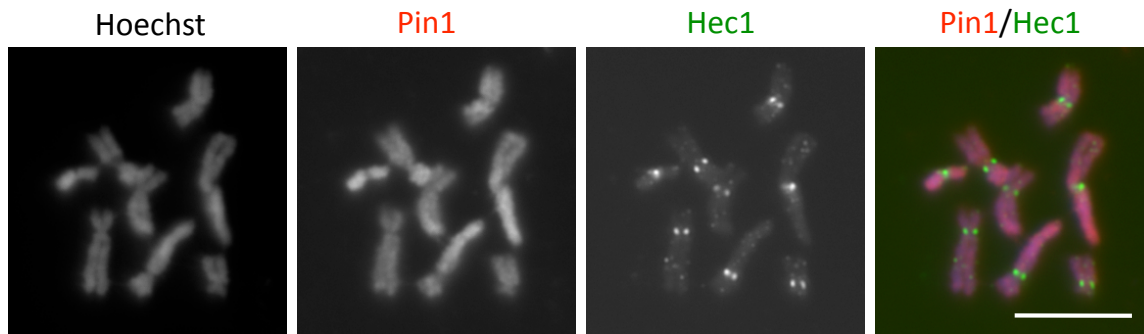


**Fig. 27: A Cdc20 variant with all Cdk1 sites mutated still localizes to kinetochores during SAC signalling, however a C-terminally truncated variant does not. (A)** Stable transgenic HeLa cell lines that express Myc-tagged wildtype Cdc20 (Cdc20<sup>WT</sup>) or a variant lacking all Cdk1 phosphorylation sites (Cdc20<sup>7A</sup>) were arrested in mitosis with a double thymidine-nocodazole block. After mitotic shake-

off, cells were harvested and transferred to fresh medium containing nocodazole plus doxycycline (+Tet) or the corresponding carrier, ethanol (-Tet). After 6 h of incubation, cells were harvested and localization as well as expression of the transgenes were analyzed by immunofluorescence and immunoblot analysis. Left panel: Immunoblot analysis of total cell lysates with anti-Myc and anti- $\alpha$ -tubulin antibodies. Right panel: Immunofluorescence pictures of the corresponding cells stained with antibodies against the kinetochore marker Hec1 (green) and the Myc epitope (red). DNA was stained with Hoechst 33342. **(B)** Schematic representation of Cdc20 variants (WT=wild type, IR= C-terminally truncated variant with last two amino acids Ile and Arg removed,  $\Delta$ C= C-terminally truncated variant with the last 29 amino acids deleted, 7A= Cdk1 phosphorylation-site deficient, BPM= Bub1 phosphorylation-site deficient). The red circles indicate the positions of mutations introduced to destroy Cdk1 or Bub1 phosphorylation sites. **(C-E)** HeLa cells were transfected with plasmids coding for different N-terminally Flag tagged Cdc20 variants (shown in B), arrested in mitosis with nocodazole and harvested by mitotic shake-off. Thereafter, cells were subjected to immunoblot and immunofluorescence analysis **(C)** Representative IF pictures of mitotic cells stained with antibodies against the Flag epitope (green) and CREST as a marker for centromeres (red). DNA was counterstained with Hoechst 33342. Scale bar is 10  $\mu$ m. **(D)** Immunoblot analysis with anti-Flag and anti- $\alpha$ -tubulin antibodies **(E)** Mitotic cells (30 each) with kinetochore-associated (localized) or no detectable Flag signal (not localized) were counted. Numbers are presented in a table.

#### **2.3.4. Pin1 localizes to chromosomes during prometaphase**

Many proteins involved in the SAC localize to the kinetochore for efficient generation of the Mad1-directed “wait anaphase” signal. A fraction of Cdc20 also localizes to kinetochores, although the basis and function of its recruitment are unknown. As Pin1 directly interacts with Cdc20, the question arises whether Pin1 co-localizes with Cdc20 during mitosis. To address this issue, 293T cells were arrested in mitosis with nocodazole. Thereafter, cells were harvested and prepared for chromosome spreads, which were followed by an immunofluorescence analysis of Pin1 and the kinetochore marker Hec1. No direct localization of Pin1 to kinetochores could be observed in any sample analyzed indicating that Pin1 does not co-localize to kinetochores. However, Pin1 was associated with the chromatin indicating close proximity of this cis-trans isomerase with the kinetochore-bound components of the SAC signalling pathway (Fig. 28).



**Fig. 28: Pin1 localizes to chromosomes in mitosis:** Chromosome spreads were prepared from mitotically arrested Hek293T cells and subjected to IF microscopy of kinetochore protein Hec1 (green) and Pin1 (red). DNA was stained with Hoechst 33342. Scale bar is 5  $\mu$ m.

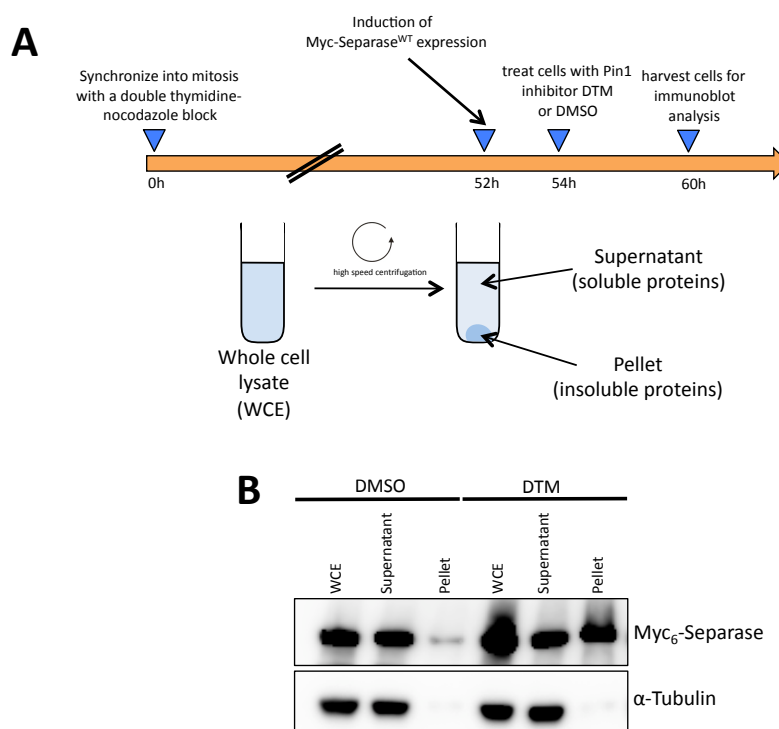
#### 2.4. A Putative role of Pin1 in separase stability during mitosis

Results in this study suggest that Pin1 has an active role in maintaining the SAC during a prolonged mitotic arrest. As Pin1 mostly interacts with mitotic phosphoproteins it is imaginable that Pin1 harbours additional, yet undiscovered mitotic functions. One of these functions could be related to separase, the protease that initiates sister chromatid segregation at the anaphase onset. Separase is phosphorylated at serine 1126 by Cdk1/cyclin B1. This phosphorylation is required for its proper inhibition by cyclin B1. This crucial phosphorylation site is followed by a proline and thus, matches the consensus site for Pin1 substrates. As a conformational switch of separase after phosphorylation on serine 1126 is postulated (Boos et al., 2008), it is possible that Pin1-dependent isomerization causes this conformational change, thereby creating a binding site for cyclin B1.

To address this issue, preliminary experiments were performed using a Hek293T cell line, which inducibly expresses Myc-tagged wildtype separase. These cells were arrested in mitosis with a double-thymidine nocodazole protocol. Upon entry into mitosis, expression of the transgene was induced by addition of doxycyclin. Subsequently, Pin1 inhibitor DTM or its solvent DMSO were added to the cells. 6 hours after incubation, cells were harvested and lysed. These lysates (WCE) contained the complete amount of soluble and insoluble proteins. A sample was detached from these lysates for immunoblot analysis. After centrifugation of these lysates, the supernatant containing only sol-

uble proteins was detached from the pellet (containing the whole amount of insoluble proteins). The pellet was solubilized by sonification in an 8 M Urea-containing buffer. These three samples of each condition were analyzed by immunoblotting using antibodies against the Myc epitope, securin and  $\alpha$ -tubulin.

Interestingly, a great increase of insoluble wildtype separase levels could be detected upon Pin1 inhibition compared to DMSO addition (Fig. 29B), indicating that Pin1 contributes to the solubility of separase during mitosis.



**Fig. 29: Pin1 might influence mitotic stability of separase: (A)** Experimental outline. Briefly, stable transgenic Hek293 cells that express Myc<sub>6</sub>-tagged wildtype separase (Myc<sub>6</sub>-Separase<sup>WT</sup>) were synchronized in mitosis using a double thymidine-nocodazole protocol. Thereafter, expression of Myc-Separase<sup>WT</sup> was induced by addition of doxycyclin and shortly afterwards, Pin1 inhibitor DTM or DMSO was added. Cells were harvested 8 h after induction and lysed using a native buffer to obtain the total quantity of soluble and insoluble proteins of the cells (WCE). A small portion of these samples was detached for immunoblot analysis. The remaining amounts of the samples were then centrifuged at high speed to separate the soluble and insoluble protein fractions from each other. After centrifugation, the supernatants (containing the soluble proteins) were detached from the pellets (containing the insoluble proteins). The pellets were washed several times with 1xPBS and solubilised by sonification in an 8 M Urea-containing buffer. Comparable amounts of these three samples of each condition were subjected to an SDS-PAGE and analyzed by immunoblot using antibodies against the Myc-epitope and  $\alpha$ -tubulin.

### 3. DISCUSSION

Results in this thesis shed light on a surprising new function of Pin1 in the maintenance of the spindle assembly checkpoint. This role is likely linked to the newly described interaction between Pin1 and the APC/C coactivator Cdc20. In the following discussion, the impact of Pin1 in checkpoint signalling and the importance of Pin1-Cdc20 binding will be discussed. Furthermore, the discrepancy between Pin1's general status as an on-cogene and the finding that Pin1 supports SAC activity will be pointed out. Moreover, the interesting finding that the last 29 amino acids of Cdc20 seem to be required for its localization to the kinetochores and its possible function during SAC signalling will be elaborated on. Finally, preliminary results of a potential SAC-independent effect of Pin1, positively affecting the stability of separase, will be discussed.

#### 3.1. Pin1 maintains the spindle assembly checkpoint

Pin1 has been shown to fulfil many pivotal functions during cell cycle progression and especially in mitosis. Consequently, the majority of Pin1 substrates are mitotically phosphorylated proteins (Shen et al., 1998). Previous reports indicated that Pin1 might have an indirect role on the duration of the SAC in prometaphase arrested cells, however a direct influence of Pin1 could not be proven (Campaner et al., 2005). No studies have been published yet showing that a SAC component is a direct target of Pin1. This circumstance might be explained with the promiscuous nature of Pin1 being involved in several cell cycle-related processes. Thus, experimental approaches cannot make use of up- or downregulation of Pin1: Overexpression leads to a G2 arrest probably due to stabilization of the APC/C inhibitor Emi1 and depletion to a G1 arrest due to inhibition of S-phase initiation (Lu et al., 1996, 2007; You et al., 2002; Bernis et al., 2007). At the beginning of this thesis, no suitable, specific Pin1-inhibitor (for cell culture purposes) was available. Thus, early experiments were performed using the *X. laevis* cell free system. Depleting endogenous Pin1 from SAC-activated extracts followed by release from the CSF-arrest resulted in an abrogation of the arrest (Fig. 12). Securin was degraded under these conditions, although a functional SAC would normally prevent its destruction.



Furthermore, there are strong indications that Pin1 also sustains prolonged SAC activity in mammalian cells: Pin1 inhibition in prometaphase arrested Hela or HCT116 cells caused premature loss of phosphorylated histone H3 and degradation of APC/C substrates (Figs. 13,14,15). Consistently, the interesting observation was made that nocodazole treated Hela cells upon Pin1 inhibition showed an abnormal phenotype similar to that of flat adherent interphase cells (Fig. 17). Furthermore, inhibition of Pin1 in mitotically arrested HCT116 partially induced tetraploidy when the inhibitor was washed out prior to S-phase (Fig. 16).

However, closed examination of the results revealed that degradation of securin in Pin1-depleted SAC activated extracts took up to 60 minutes and, thus, was slower than proteolysis of securin in untreated extract samples without established SAC. One possible explanation is that Pin1 is not an essential component of the SAC unlike Mad2, whose depletion triggers rapid APC/C<sup>Cdc20</sup> activation and degradation of SAC-sensitive substrates (Li and Benezra, 1996). Another consideration is that the presence of nocodazole and sperm nuclei of a high concentration, the extract exhibits an abnormal behaviour, with a general delay in exit into interphase. Results obtained from cell culture experiments support the model of a supporting but non-essential checkpoint function of Pin1. When adding Pin1 inhibitors to prometaphase arrested Hela and HCT116 cells, these cells still exhibited some SAC response. However, after 6-7 hours of treatment, checkpoint silencing could be observed, resulting in loss of phosphorylated histone H3 and reduction of cyclin B1/securin levels (Fig. 15). Thus, it seems that Pin1 does not contribute essentially to the establishment of the SAC but rather ensures its robust maintenance.

It is unlikely that the observed phenotypes are due to mitotic slippage. This molecular mechanism does usually not occur before  $\geq 30$  h after nocodazole addition and involves cyclin B1 degradation followed by severe micronucleation and apoptosis. In similar experiments, cyclin B1 degradation in the context of mitotic slippage did not occur before 32 h of nocodazole treatment and this degradation went along with severe micronucleation (Kim et al., 2005; Chan et al., 2008; Xu et al., 2010) In contrast, most experiments performed in the present study did not exceed 20 h of nocodazole treatment and micronucleation and enhanced levels or apoptosis were not observed.



An intact SAC is a prerequisite for genomic integrity and SAC failure often induces aneuploidy, which is a major cause of cancer (Pellman, 2007). It is possible that under certain circumstances during the process of chromosome segregation, the cell is confronted with severe problems requiring a prolonged checkpoint, for instance in the presence of genotoxic stress or spindle toxins. It has to be stated that natural duration of mitosis can extend several hours to days in some cases, although “normal” mitosis should not take more than one hour. Thus, in the case of a prolonged mitotic arrest sustained SAC activity is even more important and could emphasize Pin1’s role in SAC signalling. A comparison of the results from the *X. laevis* system and the cell culture experiments indicates that Pin1 might have a more pronounced function in amphibians where securin degradation commences minutes after  $\text{Ca}^{2+}$  addition to Pin1-depleted SAC-activated egg extracts while it started only hours after chemical inhibition of Pin1 in nocodazole-treated human cells. In mammalian systems, SAC signalling involves a few dozens players which could mask Pin1 failure better than in *X. laevis*. It is possible that the role of Pin1 in the mammalian SAC is more redundant and that other prolyl isomerases can partially replace Pin1 in regards to this function. It is further imaginable that co-depletion (or co-inhibition) of another checkpoint component of minor importance could trigger faster Pin1-dependent SAC silencing in the *Xenopus* cell free system.

In the context of a rather mild influence of Pin1 on the SAC during mammalian mitosis, the question can be raised, whether Pin1 might have a more important role in checkpoint signalling during meiosis. The SAC is also a crucial timer and regulator of murine meiosis and exhibits an important role of preventing premature DNA segregation in both meiotic divisions. Indeed, chromosome defects during meiosis cause miscarriages or trisomies (Roizen and Patterson, 2003). In contrast to prometaphase I of mouse oocytes, which starts after germinal vesicle breakdown (GVBD) and takes 6-8 hours (Terret et al., 2003), the whole process of mitosis usually does not exceed 1 h. It is therefore not surprising that SAC ensures proper length of prometaphase I. It will therefore be of interest to ask whether Pin1 influences the timing of meiosis as well. Prolonged SAC activity during prometaphase I would then be of major importance and Pin1 could potentially support continuous checkpoint signalling. Indeed, preliminary work from the Wassmann lab indicates a role of Pin1 in mammalian meiosis: Injection of Pin1

inhibitors into mouse oocytes resulted in faster progression through meiosis I and premature polar body extrusion of the first polar body (Katja Wassmann, personal communication).

### 3.2. Pin1 interacts with APC/C coactivator Cdc20 during mitosis

Given that there were several indications for Pin1 being involved in SAC functionality, the next step was to search for potential Pin1 substrates, which are involved in SAC signalling and whose Pin1-dependent inhibition could cause the observed phenotype. In this context all proteins that are directly or indirectly involved in the SAC could be regarded as potential Pin1 targets. It was already postulated that Pin1 interacts with APC/C subcomponent Cdc27. This association might have explained Pin1's influence on the SAC, since Cdc27 is required for the APC/C to target securin and cyclin B1. However, Pin1-Cdc27 interaction was only described in human cell culture, and this association could not be reproduced using *X. laevis* extracts (Shen et al., 1998; Fig. 20).

It is therefore possible that the reported Cdc27-Pin1 interaction was indirect and mediated by Pin1 substrates like Emi1 (Bernis et al., 2007) or Cdc20 (see below).

Several reasons in this thesis strongly argue for an interaction between Pin1 and Cdc20. This association was observed in human cells and *X. laevis* CSF extracts and could be reproduced in independent pulldown and IP approaches (Figs. 19,20,21). Importantly, the Cdc20-Pin1 interaction could be demonstrated by using heterologously expressed purified components (Figs. 19B, 22A). This association occurs in mitosis and is phosphorylation dependent (Figs. 19A, 22D). A Cdc20 variant (Cdc20<sup>7A</sup>), which lacked Cdk1-dependent phosphorylation sites (Yudkovsky et al., 2000), failed to associate with Pin1 but notably, retained its ability to interact with the APC/C (Fig. 23C).

It is possible that not only the binding of Pin1 to Cdc20 is required for a robust checkpoint but furthermore the subsequent Pin1-directed isomerization of Cdc20?

There are several indications that this is the case: First, inducible expression of a dominant negative Pin1 variant (Pin1<sup>DN</sup>) in prometaphase arrested cells triggered a reduction of mitotic phosphorylation of histone H3, indicative of checkpoint override (Fig. 18). This variant still binds to its substrates but lacks catalytic activity (Lu et al., 2002; Bernis et al., 2007). Moreover, Cdc20 associated with less Mad2 in mitotic 293T cells

overexpressing Pin1<sup>DN</sup> compared to wildtype Pin1 (Fig. 23B), indicating limited sequestration of Cdc20 into the MCC. Finally, in a similar approach, Cdc20<sup>7A</sup> (deficient in Cdk1-dependent phosphorylation and binding to Pin1) showed reduced association with Mad2 relative to wildtype Cdc20 (Fig. 23C). These observations are consistent with published results, as Cdk1-directed phosphorylation of Cdc20 has been reported to be indispensable for proper Mad2-dependent inhibition and required to prevent premature activation of the APC/C: In mammalian cell culture, inhibition of Cdk1 during prometaphase displaces Cdc20 from Mad2 (D'Angiolella et al., 2003). Furthermore Cdc20<sup>7A</sup> showed greater premature association with the APC/C in a prometaphase arrest (Fig. 23C) and the same variant has been reported to greatly enhance *in vitro* APC/C activity (Yudkovsky et al., 2000). Thus these data suggest that not only binding of Cdc20 to Pin1 after Cdk1-dependent phosphorylation but also subsequent isomerization might be required for maintaining the SAC during a prolonged mitotic arrest.

### 3.3. Pin1 influences mitotic distribution of Cdc20

Encouraged by the discovery of a new Pin1 substrate, the molecular consequences of Pin1 binding to Cdc20 were investigated. Some possible effects to come into consideration are outlined in Fig. 30. Pin1 acts as a molecular switch influencing a broad range of cellular processes. Consequences of Pin1-mediated isomerization are very manifold. For instance, Pin1 either promotes or prevents ubiquitylation of several substrates (Yeh et al., 2006; Siepe and Jentsch, 2009). Though, neither a possible influence of Pin1 on Cdc20 stability (Fig. 24) nor a general impact on Cdc20 ubiquitylation levels could be proven (data not shown). In this context it has to be stated that a potential influence of Pin1 on oligo-ubiquitylation of Cdc20 is unlikely as Rape and colleagues suggested that oligo-ubiquitylation promotes rapid and complete disassociation of Mad2 from Cdc20 within minutes (Reddy et al., 2007). Such strong effects could never be observed and also the long duration of the prometaphase arrest during Pin1 inhibition prior the SAC override speaks against the theory Pin1 influencing oligo-ubiquitylation of Cdc20 and rapid disassociation from Mad2. The general weak impact of Pin1 on the SAC speaks rather for a non-essential function, which is responsible for maintaining a prolonged SAC arrest.

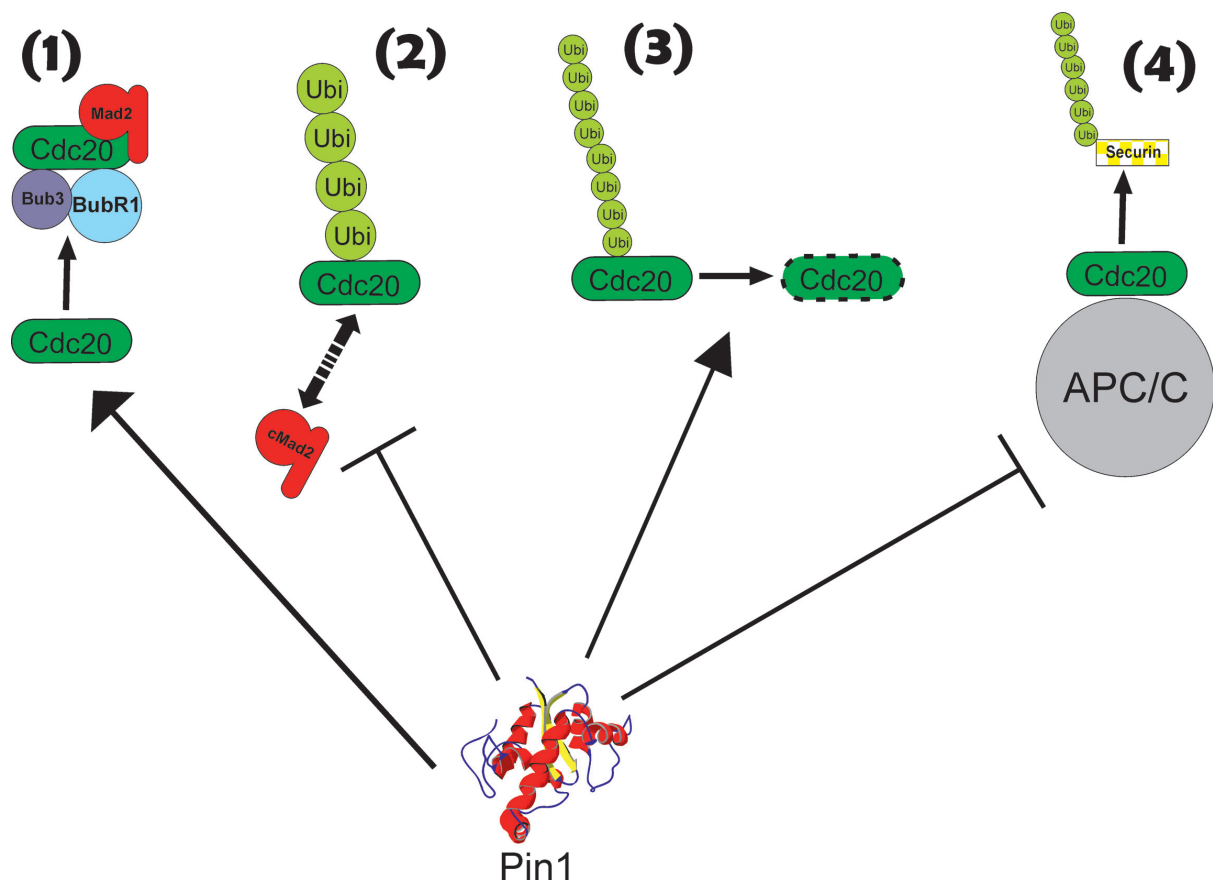
Interestingly, upon Pin1 inhibition in 293T cells, the observation could be made that Cdc20 changes its molecular distribution from mainly associated with Mad2 to primarily associated with the APC/C (Fig. 23). There are several studies claiming that upon anaphase onset Cdc20 preferentially associates with APC/C component Cdc27 (Izawa and Pines, 2011). Moreover similar effects could be demonstrated when performing an IP of endogenous Cdc20 or Cdc27 using 293T cells overexpressing a dominant negative variant of Pin1 or treated with the Pin1 inhibitor DTM (Fig. 23). These effects suggest a rather general impact of Pin1 on Cdc20 distribution during prometaphase causing a weakened association of Cdc20 with Cdc27 and hence, the APC/C (Fig. 31).

Cdc20 is known to bind at least five different APC/C subunits. Which of these is the preferred docking site of Cdc20 depends on the current cell cycle stage. An important understanding of how Cdc20 activates the APC/C upon anaphase onset comes from the Pines laboratory. Recently, a new interaction between Cdc20 and the APC/C subunit APC8 has been described. This interaction occurs both in prometaphase and anaphase, Upon anaphase onset this interaction is weakened indicating that it more important for SAC signalling and less important for anaphase activity of the APC/C. This interaction seems to be important for the APC/C to distinguish between prometaphase substrates like Nek2A and cyclin A and SAC-sensitive substrates like securin (Izawa and Pines, 2011). Hence, it was postulated that Pin1 might stimulate APC8-Cdc20 binding.

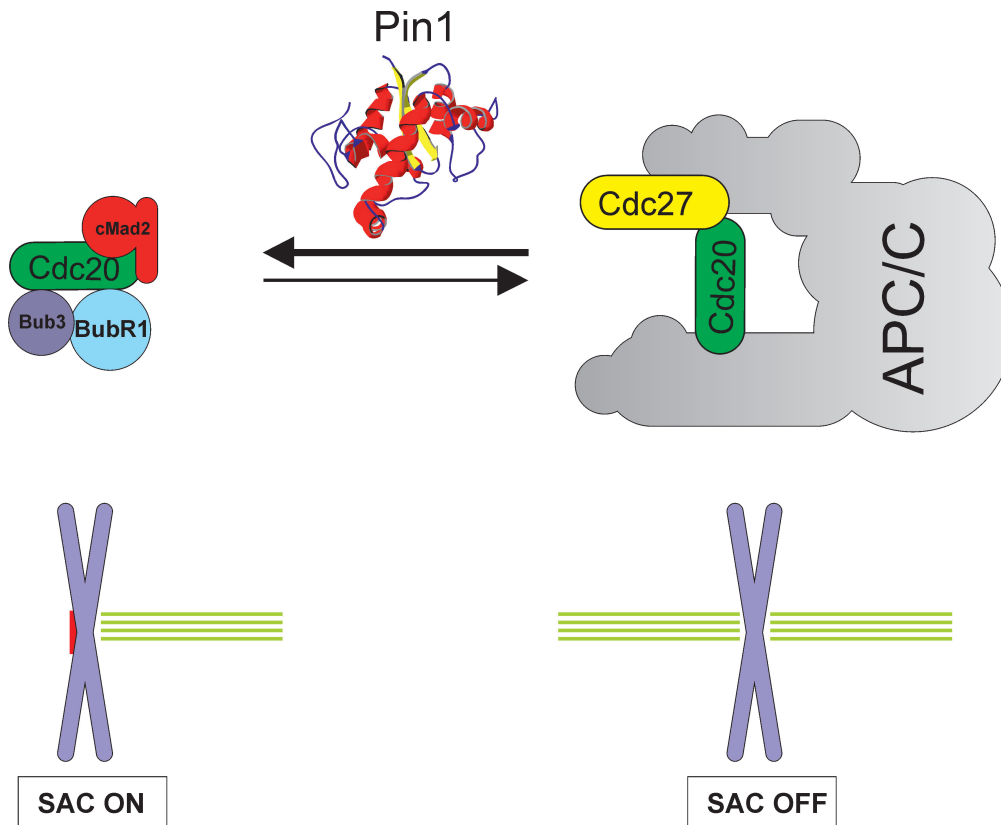
Unfortunately, it was not possible to experimentally validate an influence of Pin1 on Cdc20-APC8 binding. Due to experimental difficulties, clear conclusions can only be made in association studies using purified components. This is, however, difficult, as it cannot be ruled out that additional co-factors are required.

As there were no clear indications that Pin1 stimulates or prevents associations of Cdc20 with specific subunits of the APC/C the question was raised, whether it is also possible that Pin1 has an impact on MCC formation or stability. It has been shown that Cdc20-Mad2 association is strongly decreased upon Cdk1 inhibition (D'Angiolella et al., 2003). Thus, Pin1 might stimulate this Cdk1-directed association of Cdc20 with Mad2. However, *in vitro* experiments using recombinant Cdk1 kinase, Mad2, Cdc20 and Pin1 did not exhibit a clear dependence of the Mad2-Cdc20 binding on Pin1 activity leaving this question unanswered (data not shown). *In vivo*, Mad2 binding to Cdc20 is strongly

decreased upon Pin1 inhibition or Pin1<sup>DN</sup> overexpression in nocodazole-arrested 293T cells (Fig. 23). However, also in this approach it cannot be decided whether decreased Mad2-Cdc20 binding is a direct effect caused by Pin1 inhibition or a general consequence of SAC silencing. In conclusion, further experiments using more sophisticated approaches will allow insight in the exact molecular mechanism of Pin1-mediated SAC maintenance.



**Fig. 30: Possible influence of Pin1 on Cdc20:** Several consequences of Pin1-mediated isomerization of Cdc20 are imaginable. (I) Pin1 stimulates Mad2-directed turnover of Cdc20 into the MCC. (II) Pin1 prevents oligo-ubiquitylation of Cdc20 ensuring stable association with Mad2. (III) Pin1 stimulates prometaphase degradation of Cdc20 ensuring prolonged SAC activity. (IV) Pin1 prevents premature association of Cdc20 with the APC/C via Cdc27.



**Fig. 31: Model of general impact of Pin1 on Cdc20 distribution during SAC signalling:** Pin1 is responsible for keeping Cdc20 in check. It is postulated that during SAC activity (SAC ON) Pin1 mediates turnover of Cdc20 into the MCC and prevents Cdc27-Cdc20 association and premature APC/C activation until not all chromosomes are properly aligned to the mitotic spindle. Upon anaphase onset, Cdc20 serves as a co-activator of the APC/C to sequester the separase inhibitors cyclin B1 and securin (SAC OFF).

### 3.4. Kinetochoric Cdc20 distribution during prometaphase

Besides a decrease of phosphorylated histone H3, securin and cyclin B1 in prometaphase arrested cells, several other observations could be made upon Pin1 inhibition/depletion. One of them was the loss of Cdc20 from kinetochores (Figs. 25,26). However, experiments performed in this thesis suggest rather an indirect influence of Pin1 on the pattern of Cdc20 localization in mitosis: A transiently overexpressed non-Pin1 binding variant of Cdc20 (Cdc20<sup>7A</sup>) still accumulated at kinetochores in nocodazole arrested HeLa cells. Also mild transient expression of this variant in stable transgenic HeLa cells upon entry into mitosis didn't affect its ability to localize to kinetochores (Fig. 27A,C). It has been proposed, that this specific distribution of Cdc20 is indispensable for

proper SAC functionality and that the WD40-repeats of Cdc20 mediate kinetochoric association (Kallio et al., 2002). Data of this study suggest that not only the WD40 repeats but the last 29 amino acids of Cdc20 seem to be chiefly responsible for localization: A C-terminally truncated variant of Cdc20 showed no distinct localization during prometaphase (Fig. 27). Close examinations of different Cdc20 variants indicated, that kinetochore localization is independent of Cdc27: A Cdc20 variant lacking the last two amino acids (IR) still localized to kinetochores but was no longer able to interact with Cdc27. Furthermore, it seems that localization is not driven by phosphorylation, as Bub1- or Cdk1-phosphorylation deficient Cdc20 variants were not altered in their ability to localize to kinetochores (Fig. 27). It is possible that localization does not require any additional cofactors.

How might Cdc20 localization contribute to the SAC? It is imaginable that only free Cdc20 localizes to kinetochores leading to rapid association with nearby activated Mad2. Another possibility is that kinetochore associated Cdc20 is more readily available as a premature substrate of the APC/C. As several studies indicate APC/C-dependent degradation of free Cdc20 as a prerequisite for accurate and prolonged SAC activity (Nilsson et al., 2008), it is possible that kinetochoric localization of Cdc20 triggers its association with the APC/C. Consistently, the APC/C also been shown to localize to kinetochores during mitosis (Vigneron et al., 2004) indicating that the E3 ligase and its co-activator are in closed proximity during SAC activity. Furthermore, several other checkpoint components also localize to kinetochores. Checkpoint kinases like Bub1 or MAPK might contribute to proper Cdc20 phosphorylation and prevent premature activation of the APC/C (Sharp-Baker and Chen, 2001). However, it has to be mentioned that there is no clear evidence yet that Cdc20 localization is a precondition for proper checkpoint function. It is conceivable that localization contributes to rapid SAC silencing and APC/C depression. First, Cdc20<sup>7A</sup> and Cdc20<sup>BPM</sup>, Cdc20 variants that cannot be phosphorylated by Cdk1 and Bub1, respectively, still localize to kinetochores (Fig. 27). For Cdc20<sup>7A</sup>, it is known that it preferentially associates with the APC/C (Fig. 22) and greatly enhances its ligase activity (Yudkovsky et al., 2000). Furthermore, expression of a Bub1-phosphorylation deficient Cdc20 variant in prometaphase arrested Hela cells triggers checkpoint override and partial tetraploidy (Tang et al., 2004). Therefore, it is

also possible that kinetochoric Cdc20 associates with APC/C upon checkpoint inactivation ensuring fast degradation of APC/C substrates. Supporting this thesis is that both Bub3 and BubR1 show localization to kinetochores during prometaphase (Vigneron et al., 2004) indicating that not free Cdc20 but the MCC localizes to kinetochores. However, it has been stated that MCC formation does not require kinetochores (Fraschini et al., 2001). It is, possible that Cdc20 localization at the kinetochores ensures its rapid association with Mad2 enabling SAC engagement and fast and reliable activation of the APC/C upon anaphase onset. In conclusion, these two theses do not mutually exclude each other.

### **3.5. Pin1 and cancer**

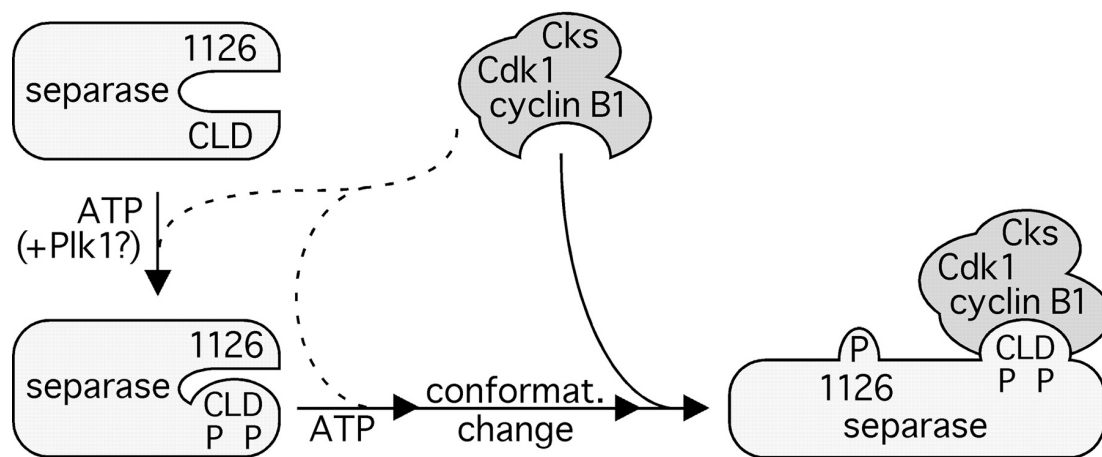
It has been shown that Pin1 is overexpressed in many cancer cells (Xu and Etzkorn, 2009) and that the level of overexpression correlates with the clinical outcome of the patient. In some reports Pin1 is suggested as an oncogene (Kuramochi et al., 2006; Lu and Zhou, 2007). Therefore, it seems to be a discrepancy that Pin1 supports SAC activity, especially during a prolonged prometaphase arrest, thus ensuring proper genomic stability. This seeming conflict might be explained with the aggressive nature of cancer cells in general. In this context it has to be stated that almost all cancer cells contain deregulated spindle assembly checkpoint components, for instance in most breast cancer cells Bub3 expression is frequently deregulated in breast cancer (Enders, 2010). A promising hypothesis is that in these cases Pin1 activity wouldn't have any positive impact on SAC maintenance as the SAC is severely damaged. Genomic instability, high rate of mutation, deregulation of several oncogenes and downregulation of tumor suppressors in advanced stages of cancer wouldn't be balanced by Pin1's positive impact on SAC functionality anymore. It is further imaginable that the functions of Pin1 in cancer cells differ from those in healthy tissues: Pin1 is an important regulator of mitotic entry and Pin1 overexpression would normally lead to a G2 arrest. However, Pin1's general ability to control cell cycle progression could be perturbed and Pin1 might gain new functions supporting cell growth and aggressiveness in malignantly transformed cells. The exact role of Pin1 in growing tumour cells, however, still remains controversial.



### 3.6. Possible influence of Pin1 on separase stability

During SAC signalling, separase activity is obstructed by its mutually exclusive inhibitors securin and cyclin B1. Inhibitory binding of the Cdk1/cyclin B1 complex to the CLD domain of separase requires Cdk1-dependent phosphorylation of serine 1126 followed by a presumed conformational switch (Fig. 32; Holland and Taylor, 2006; Boos et al., 2008). The crucial serine 1126 phosphorylation site is followed by a proline and, thus, matches the consensus site for Pin1 substrates. Therefore Pin1-dependent isomerization might catalyze this predicted conformational change, thereby creating a binding site for cyclin B1. Indeed, preliminary experiments indicate that Pin1 might indeed trigger this switch. Upon treatment of prometaphase arrested Hek293 cells with Pin1 inhibitor DTM, inducibly expressed wild-type separase is generally found insoluble compared to DMSO treated cells (Fig. 29B). It is imaginable that after Cdk1-dependent phosphorylation of separase, Pin1 mediates a catalytic switch thus enabling the binding of the Cdk1/cyclin B1 complex to separase, promoting its stabilization (Fig. 32). In accordance, cyclin B1 binding to separase seems to have, similar to securin, a positive influence on the stability of separase (S. Hellmuth, personal communication). It is postulated that without Pin1 isomerization, Cdk1/cyclin B1 cannot bind to separase after Cdk1-dependent phosphorylation and hence, separase would tend to aggregate. Therefore, Pin1-mediated isomerization could be an additional mechanism in regulating mitotic separase activity and stability.

Finally, additional experiments to get more insight into the putative Pin1-mediated isomerization of separase are inevitable. Further aggregation assays and cyclin B1/separase binding studies upon Pin1 inhibition or overexpression have to be performed.



**Fig. 32: Conformational switch of separase after Cdk1 phosphorylation on serine 1126 might enhance cyclin B1 binding:** Phosphorylation of serine 1126 probably induces a conformational switch enhancing binding of cyclin B1 to the CLD-domain of human separase. It is possible, that the prolyl isomerase Pin1 mediates this conformational switch. Figure taken from Boos et al. (2008).

### 3.7. Conclusion

It could be shown that Pin1 has a positive impact on SAC maintenance and interacts with the APC/C co-factor Cdc20 in a mitosis specific manner. Pin1 sequestration phosphorylated of Cdc20 into the MCC or prevents its association with the APC/C or both. The data obtained in this study suggests that Pin1 is a novel component of the SAC ensuring its robustness. During unperturbed cell cycle progression, when chromosome alignment occurs efficiently, Pin1-mediated influence on SAC activity seems to be negligible. However, Pin1 could be of major importance in situations, in which the cell is forced to arrest in mitosis. Furthermore, it is possible that Pin1 exhibits a more critical role in checkpoint signalling during meiosis I. Future experiments have to scrutinise the exact mechanism of the Pin1-mediated impact on SAC maintenance.

## **4. MATERIALS AND METHODS**

If not indicated otherwise, companies are situated in Germany.

### **4.1. Materials**

#### **4.1.1. *Hard and Software***

Chemiluminescence signals of Western blots and Coomassie stained gels were digitized using an "LAS-4000" or an "LAS-3000" system, respectively (Fuji Film Europe, Düsseldorf; now GE Healthcare, USA). The image analysis software MultiGauge (Fuji Film Europe, Düsseldorf) was used to export raw data obtained from "LAS-3000" and "LAS-4000" systems. Image processing was performed with "Adobe Photoshop CS3" (Adobe Systems Inc., San Jose, CA, USA). Figures were generated using "Microsoft PowerPoint 2008", "Microsoft Excel 2008" (both Microsoft Corporation, Redmond, WA, USA) and CorelDraw 11 (Corel Corp, Ottawa, Kanada). "DNASTAR Lasergene" (GATC Biotech, Konstanz) was used for analysis of DNA and protein sequences. Literature and database searches were done with electronic online services provided by the "National Center for Biotechnology Information" (<http://www.ncbi.nlm.nih.gov/>). The Pin1 structure used in Fig. 8 & Fig. 31 was generated using the QUARK ab initio services: (<http://zhanglab.ccmb.med.umich.edu/QUARK>). A service of the European Bioinformatics Institute was used for sequence alignments ("EMBOSS Pairwise alignment" algorithm, <http://www.ebi.ac.uk/Tools/emboss/align/index.html>).

#### **4.1.2. *Protocols***

The methods described in this section are based on standard techniques or followed by the manufacturer's instructions. Where protocols have been modified, detailed information is provided. For all methods, de-ionized sterile water and, when appropriate, sterile solutions and sterile flasks were used.

#### **4.1.3. *Chemicals and reagents***

Unless otherwise stated, chemicals and reagents (pro analysis grade) were purchased

from AppliChem (Darmstadt), Biomol (Hamburg), Biorad (Munich), GE Healthcare (Munich), Fermentas (St. Leon-Rot), Invitrogen (via Fisher Scientific, Schwerte), Merck/Calbiochem (Darmstadt), Millipore (Schwalbach), New England Biolabs (NEB, Frankfurt a. M.), Pierce/Fisher Scientific (Schwerte), Promega (Mannheim), Qiagen (Hilden), Roche Diagnostics (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) and Sigma-Aldrich (Steinheim).

#### **4.1.4. Antibodies**

Commercial antibodies and affinity matrices used in this study were as follows: mouse monoclonal anti-securin (clone DCS-280, Abcam, Cambridge, UK), mouse monoclonal anti-Flag (clone M2, Sigma-Aldrich), rabbit polyclonal anti-Flag (Sigma-Aldrich), mouse monoclonal anti-Cdc20 (clone E-7, Santa Cruz), mouse monoclonal anti-Mad2 (clone 17D10, Santa Cruz), rabbit polyclonal anti-Bub3 (Sigma Aldrich), rabbit polyclonal anti-phospho histone H3 (Millipore), mouse monoclonal anti-phospho histone H3 (clone AH3-120, Sigma Aldrich), rabbit polyclonal anti-Myc (Santa Cruz), rabbit polyclonal anti-cyclin B1 (Santa Cruz), CREST human autoserum (Immunovision, Springdale, USA), mouse monoclonal anti-Bub1 (clone 14H5, Sigma Aldrich) and mouse monoclonal anti-Hec1 (clone 9G3.23, Genetex, Irvine, CA, USA).

Non-commercial antibodies used were: rabbit polyclonal anti-pSX104 (against hSecurin), rabbit polyclonal anti-xPin1, rabbit polyclonal anti-xCdc20, rabbit polyclonal anti-Mad2, goat polyclonal anti-Cdc27 (gift from Thomas Mayer, Konstanz), sheep polyclonal anti-BubR1 (gift from Steven Taylor, Manchester), goat polyclonal anti-GST, mouse monoclonal anti-Myc (clone 4A6, Developmental Studies Hybridoma Bank), mouse monoclonal anti- $\alpha$ -tubulin (clone 12G10, Developmental Studies Hybridoma Bank).

Polyclonal goat anti-rabbit-IgG, rabbit anti-sheep-IgG, goat anti-mouse-IgG, bovine anti-goat IgG coupled to peroxidase (Sigma-Aldrich) or goat anti-mouse IgG (H or L) coupled to peroxidase (KPL, Gaithersburg, MD, USA, heavy-chain specific) were used for immunoblotting.

Unspecific rabbit IgG (Bethyl), unspecific goat IgG (Sigma-Aldrich) and unspecific mouse IgG (Sigma-Aldrich) were used as mock controls in immunoprecipitation experiments.

Cy3 goat anti-mouse IgG (H+L) (Invitrogen, USA) was used for flow cytometry analysis. Alexa Flour 488 goat anti-mouse IgG, Alexa Flour 546 goat anti-mouse IgG, Alexa Flour 546 goat anti-rabbit IgG (Invitrogen USA), Cy3 goat anti-rabbit IgG (dianova, Hamburg) and Cy3 goat anti-human IgG (Bethyl) were used for secondary detection in immunofluorescence.

#### 4.1.5. Plasmids

Vector	Origin
pCS2	(Turner and Weintraub, 1994) with modified MCS (FseI, AscI sites introduced)
pcDNA5-FRT-T0	(Invitrogen, USA) with modified MCS (FseI/AscI sites inserted)
pGEX	(GE Healthcare, USA) with modified MCS (FseI/AscI sites inserted)
pET28M	(Genomex, USA) with modified MCS (FseI/AscI sites inserted)

Plasmid No.	Insert	Tag	Backbone
pBA1980	xPin1	N-GST	pGEX
pBA2101	xPin1 <sup>S16A, C109A</sup> = xPin1 <sup>DN</sup>	N-His <sub>6</sub> -SUMO3	pET
pBA2149	hPin1	N-GST-Tev	pGEX
pBA2206	xPin1	N-His <sub>6</sub> -SUMO <sub>3</sub>	pET
pBA2320	hCdc20 <sup>A471-499</sup> = hCdc20ΔC	N-Myc <sub>6</sub> -Tev <sub>2</sub>	pCS2
pBA2321	xCdc20 <sup>A478-506</sup> = xCdc20ΔC	-	pCS2
pBA2348	hCdc20 <sup>A471-499</sup> = hCdc20ΔC	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2349	hPin1	N-Myc <sub>6</sub> -Tev <sub>2</sub>	pCS2
pBA2350	hPin1 <sup>S16A C113A</sup> = hPin1 <sup>DN</sup>	N-Myc <sub>6</sub> -Tev <sub>2</sub>	pCS2
pBA2351	hCdc27	N-Myc <sub>6</sub> -Tev <sub>2</sub>	pCS2
pBA2353	hCdc20	N-His <sub>6</sub>	pCS2
pBA2372	hPin1 <sup>S16E</sup> = hPin1 <sup>NB</sup>	N-GST-Tev	pGEX
pBA2463	Cdc20 <sup>1-144</sup>	N-His <sub>6</sub> -SUMO <sub>3</sub>	pET
pBA2464	Cdc20 <sup>5A; 1-144</sup>	N-His <sub>6</sub> -SUMO <sub>3</sub>	pET
pBA2484	hCdc20	N-Myc <sub>6</sub> -Tev <sub>2</sub>	pcDNA5
pBA2485	hCdc20 <sup>7A</sup> (Yudkovsky et al., 2000)	N-Myc <sub>6</sub> -Tev <sub>2</sub>	pcDNA5
pBA2524	hCdc20 <sup>7A</sup> (Yudkovsky et al., 2000)	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2549	hCdc20 <sup>S41,72,92,153,157,161A</sup> = hCdc20 <sup>BPM</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2600	hPin1	N-GST-Tev <sub>3</sub>	pCS2
pBA2705	hCdc20 <sup>175-499</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2

pBA2712	hCdc20 <sup>1-174</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2722	hCdc20	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2730	hCdc20 <sup>ΔIR</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2879	hCdc20 <sup>S41A</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2880	hCdc20 <sup>T70A</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pBA2881	hCdc20 <sup>S41A, T70A</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pCS2
pFB1280.1	xCdc20	-	pCS2
pBA2911	hPin1 <sup>WT</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pcDNA5
pBA2912	hPin1 <sup>S16A C113A</sup> = hPin1 <sup>DN</sup>	N-Flag <sub>3</sub> -Tev <sub>2</sub>	pcDNA5
No. 186 "Genes from others"	Ubiquitin	N-HA	pBS-SK- +CMV & SV40pA

Source: pBA: this study; pFB: Fransiska Böttger, AG Stemmann; No 186 "Genes from others": Stefan Müller, University of Frankfurt

#### 4.1.6. DNA oligonucleotides

##### Pin1

Primer	Sequence
5F_xPin1_BA	5'-TAATAGGCCGCGCCGATGGCGGACGAGGAGAAGCT-3'
3A_xPin1_BA	5'-GGCGCGCCTCACTCAGTGCGGAGGATAATGTGAA-3'
hPin1-5Fse	5'-AATGGCCGGCCTATGGCGGACGAGGAGAAGCTG-3'
hPin1-3Asc	5'-TTAGGCGCGCCTCACTCAGTGCGGAGGATGATG-3'
3'hPin1_aa54	5'-GGCGCGCCCTCACCTGGCAGGCTCCC-3'
5'hPin1_aa44-	5'-TAATAGGCCGCGCCAGGTGGCAAAAAACGGGCAGG-3'
BA_hPin1S16A_5'	5'-CGCCCGGGTGGGAGAAGCGCATGGCCCGCAGC-3'
BA_hPin1S16A_3'	5'-GCTGCGGGCCATGCGCTTCTCCCACCCGGGCG-3'
BA_hPin1C113A_5'	5'-AGTTCAGCGACGCCAGCTCAGCCAAGGCCAGGGG-3'
BA_hPin1C113A_3'	5'-CCCCTGGCCTTGGCTGAGCTGGCGTCGCTGAACT-3'
xPin1_S16A_BA	5'-GAAGCGAATGGCCCGAAGCTCTGGG-3'
xPin1_S16Arc_BA	5'-CCCAGAGCTTCGGGCCATTCGCTTC-3'
xPin1_C109A_BA	5'-CAGTTCAGTGACGCCAGCTCAGCTAAGGC-3'
xPin1_C109Arc_BA	5'-GCCTTAGCTGAGCTGGCGTCACTGAACTG-3'
hPin1_S16E_for	5'-TGGGAGAAGCGCATGGAACGCAGCTCAGGC-3'
hPin1_S16E_rev	5'-GCCTGAGCTGCGTTCCATGCGCTTCTCCCA-3'

##### Cdc20

Primer	Sequence
OS_hCdc20_5F	5'-TTAGGCCGCGCCCATGGCACAGTTCGCGTT-3'
OS_hCdc20_3A	5'-TTGGCGCGCCTAGCGGATGCCTTGGTGGATGA-3'
hCdc20_41A_OS	5'-CCCGGCCCGCGACCCATGCGGG-3'
hCdc20_41A_rev	5'-CCCGCATGGGTGCGGGGGCCGGG-3'
hCdc20_70A_OS	5'-GGTTCAGACCGCTCCTAGCAAACC-3'
hCdc20_70A rev	5'-GGTTTGCTAGGAGCGGTCTGAACC-3'
hCdc20_S72A_for	5'-AGACCACTCCTGCCAAACCTGGCG-3'
hCdc20_S72A_rev	5'-CGCCAGGTTTGGCAGGAGTGGTCT-3'

hCdc20_S92A_for	5'-GGAGGTGGCCGCCTTCCTCCTG-3'
hCdc20_S92A_rev	5'-CAGGAGGAAGGCGGCCACCTCC-3'
hCdc20_S153A_f	5'-AAAGTACTCTACGCCCCAAAAGGCC-3'
hCdc20_S153A_rc	5'-GGCCTTTTGGGCGTAGAGTACTTT-3'
hCdc20_S161A_f	5'-CTCCTGGCTCCGCCCCGAAGACCT-3'
hCdc20_S161A_rc	5'-AGGTCTTCCGGGCGGAGCCAGGAG-3'
Cdc20_S408A_for	5'-CTCCATCCTCTGGGCTCCCCATTACAAG-3'
Cdc20_S408A_rev	5'-CTTGTAATGGGGAGCCCAGAGGATGGAG-3'
hCdc20_TriplA_f	5'-TACGCCCCAAAAGCCGCTCCTGGCTCCGCCCCG-3'
hCdc20_TriplA_r	5'-CGGGCGGAGCCAGGAGCGGCCTTTTGGGCGTA-3'
Cdc20_S408A_for	5'-CTCCATCCTCTGGGCTCCCCATTACAAG-3'
Cdc20_S408A_rev	5'-CTTGTAATGGGGAGCCCAGAGGATGGAG-3'
hCdc20_452A_OS	5'-GAGTCTGACCATGGCCCCAGATGGGGCCA-3'
hCdc20_55_59A_OS	5'-GCGCCGGCAGGGCTCCGGGCGGAGCTCCTGGCAAATCC-3'
hCdc20_157A_OS	5'-CCAAAAGGCCGCTCCTGGCTCCA-3'
hCdc20_157A_rev	5'-TGGAGCCAGGAGCGGCCTTTTGG-3'
3'Cdc20-IR_BA	5'-GGCGCGCCTCAGCCTTGGTGGATGAGGCTGCT-3'
BA_Cdc20(del)C_3'	5'-TGGCGCGCCTCACCATAGCCTCAGGGTGTCTATC-3'
5'F_CDC20_145-e	5'-TAATAGGCCGGCCGCGAGAACAGACTGAAAGT-3'
5'F hCdc20_175-e	5'-AATGGCCGGCCGATCCTGGATGCGCCTGAA-3'
xCdc20_5F_OS	5'-GTGGCGCGCCTCATCGGATGCTTTGGTGAAT-3'
xCdc20_3A_OS	5'-TGAGGCCGGCCCATGGCACAGTTTGCATTTG-3'

#### Others

Primer	Sequence
hCdc27_5F_OS	5'-TAAGGCCGGCCGAGGATGACGGTGCTGCAGGAA-3'
hCdc27_3opA_OS	5'-TGGCGCGCCCAAATTCATCACTTTCAGCTGCATG-3'
CS2_for	5'-CGCCATTCTGCCTGGGGAC-3'
CS2_rev	5'-TCTGGATCTACGTAATACGAC-3'
GST_for	5'-CAAGCCACGTTTGGTGGTGG-3'
GST_rev	5'-CTTCAAGATATTCAAAAAGAAGT-3'

#### 4.1.7. dsRNA oligonucleotides

Target mRNA	siRNA target sequence
Pin1M	5'-GCCAUUUGAAGACGCCUCG-3'
GL2	5'-CGUACGCGGAUACUUCGAUU-3'

## 4.2. Microbiological methods

### 4.2.1. *E. coli* media

LB medium: 1% (w/v) tryptone (Difco, BD Biosciences, Heidelberg)  
0.5% (w/v) yeast extract (Difco)  
1% NaCl (w/v) dissolved in ddH<sub>2</sub>O and sterilized by autoclaving

LB agar: LB-medium with 1.5% agar (Roth, Karlsruhe)

### 4.2.2. *E. coli* strains

XL1-Blue: *E. coli* supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1, lac [F' pro AB lacI<sup>q</sup>, Lac Zdm15, Tn10 (Tet<sup>r</sup>)]  
Stratagene/AgilentvTechnologies, Santa Clara, CA, USA

Rosetta 2: *E. coli* F, ompT, hsdS<sub>B</sub> (r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>), gal, dcm, λ, Cam<sup>R</sup>  
(Novagen/Merck, Darmstadt)

Rosetta 2 (DE3): *E. coli* F, ompT, hsdS<sub>B</sub> (r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>), gal, dcm, λ  
(DE3 [lacI, lacUV5-T7 gene 1, ind1, sam7, nin5]) Cam<sup>R</sup>  
(Novagen/Merck, Darmstadt)

### 4.2.3. Cultivation of *E. coli*

*E. coli* strains were grown in Erlenmeyer flasks containing LB medium by shaking at 200 rpm at 37°C, LB agar plates were incubated at 37°C. Antibiotics for selection of transformed bacteria were added to LB media at a final concentration of 100 µg/ml (ampicillin) or 30 µg/ml (kanamycin). Culture densities were determined by measuring the absorbance at a wavelength of 600 nm (OD<sub>600</sub>) using an OD600 DiluPhotometer (Implen, München).



**4.2.4. Preparation of chemically competent *E. coli* cells**

Tbf1 buffer:	30 mM KAc	Tbf2 buffer:	10 mM MOPS/NaOH
	50 mM MnCl <sub>2</sub>		75 mM CaCl <sub>2</sub>
	100 mM KCl		10 mM KCl
	15% glycerol		15% glycerol
	pH adjusted to 5.8		pH adjusted to 7.0

For preparation of chemical-competent bacteria, 300 ml LB medium was inoculated with 4 ml of an overnight culture derived from one single *E. coli* colony and grown at 37°C until the OD<sub>600</sub> reached a value of 0.5. After chilling the culture flask on ice for 15 min, cells were pelleted by centrifugation (4°C, 5000 g, 15 min). All following steps were performed with materials and solutions prechilled to a temperature of 4°C. Pelleted bacteria were resuspended in 90 ml Tbf1 buffer and chilled on ice for 15 min. After a second centrifugation step (4°C, 5000 g, 15 min), bacteria were resuspended in 15 ml Tbf2 buffer and chilled on ice for 5 min. Finally, suspension of bacteria was aliquoted, snap-frozen and stored at -80°C prior to use.

**4.2.5. Transformation of chemically competent *E. coli***

Competent bacteria (preparation see chapter 4.2.4) were thawed on ice. For chemical transformation, 0.1-1 µl of plasmid DNA or 5 µl of a ligation reaction were added to 50 µl of bacteria and incubated on ice for 30 min. After a short heat shock for 45 s at 42°C, the cell suspension was incubated on ice for 2 min followed by addition of 500 µl LB medium and incubation at 37 °C for 45 min (for vectors carrying ampicillin resistance) or 90 min (for vectors carrying kanamycin resistance). Transformation was completed by transferring the cell suspension to LB agar plates containing the desired antibiotic (see 4.2.3). Incubation was carried out over night at 37°C.

**4.2.6. Expression of recombinant proteins in *E. coli***

For bacterial expression of GST- or His<sub>6</sub>-SUMO3-tagged proteins, chemically competent *E. coli* strains “Rosetta” or “Rosetta DE3”, respectively, were transformed with plasmids carrying the desired transgene. LB medium was inoculated with a 1:100 dilution of an

overnight culture from a freshly transformed colony. LB medium was supplemented with the desired antibiotic. The culture was grown at 37°C and expression of proteins(s) was induced by addition of IPTG (0.2 mM final concentration) at an OD<sub>600</sub> of 0.5. After shaking for 2-3 hours (depending on the size of the transgene) at 37°C, cells were harvested by centrifugation and cell pellets were either processed immediately or shock-frozen and stored at -80°C for later use.

### **4.3. Molecular biological Methods**

#### **4.3.1. Isolation of plasmid-DNA from *E. coli***

5 ml of LB medium containing the appropriate antibiotic was inoculated with a single *E. coli* XL1-Blue colony harboring the desired DNA plasmid and shaken for 8-14 h or overnight at 37°C. Plasmid-DNA was purified via alkaline lysis of the bacteria followed by isolation by anion exchange columns according to the manufacturer's instructions (Qiagen, "Plasmid Purification Handbook, Plasmid Mini Kit" or Fermentas "GeneJET™ Plasmid Miniprep Kit"). For larger amounts of plasmid DNA, 50-500 ml LB medium was inoculated as described above. Plasmid purification was performed according to the manufacturer's instructions (Qiagen, "Plasmid Purification Handbook").

#### **4.3.2. Determination of DNA concentration in solution**

DNA concentrations were measured using the ND-1000 Spectrophotometer (Peqlab, Erlangen). After applying 1 µl of a DNA containing solution to the pipetting surface of the device, the DNA concentration was determined by measuring the absorbance at a wavelength of 260 nm. An OD<sub>260</sub> = 1 equals a concentration of 50 µg/ml double-stranded DNA.

#### **4.3.3. Restriction digestion of DNA**

Sequence-specific cleavage of DNA with restriction enzymes was performed according to the instructions of the manufacturer (New England Biolabs, USA). For analytical purposes, 1 unit of a specific restriction enzyme was used for digestion of 500 ng DNA. For preparative purposes, 10 units were used for cleaving 5 µg DNA. Reaction samples were incubated in the appropriate buffer at the recommended temperature for 1-3 h. For di-

gestions of DNA with two or more different enzymes, a buffer was chosen with the highest fidelity for all enzymes. To enhance *in vitro* activity of certain restriction enzymes, 1xBSA (New England Biolabs, USA) was supplemented to the reaction buffer.

#### **4.3.4. Dephosphorylation of DNA fragments**

For prevention of religation of linearized vectors during a ligation process, the 5' end of vector DNA was dephosphorylated by adding 3 units of shrimp alkaline phosphatase and the appropriate buffer concentrate (Roche, Mannheim) followed by incubation for 30 min at 37°C. Shrimp alkaline phosphatase was heat-inactivated for 10 min at 70°C.

#### **4.3.5. Separation of DNA fragments by agarose gel electrophoresis**

TAE buffer:	40 mM Tris Base	6xDNA-loading buffer:	10 mM Tris-HCl (pH 7.6)
	20 mM acetic acid		0.03% bromphenol blue
	1 mM EDTA		0.03% xylene cyanol FF
			60% glycerol
			60 mM EDTA

For analysis and preparative isolation, DNA fragments were electrophoretically separated on agarose gels (0.8-2.0% of agarose in TAE buffer) containing ethidium bromide (1 µg/ml final concentration). DNA samples were mixed with 6xDNA loading buffer and separated at 120 V in TAE buffer in an electrophoresis chamber (Hoefer). DNA fragments were visualized using an UV transilluminator (Syngene, UK). The size of the obtained fragments was estimated by standard size markers (O'GeneRuler 1 kb or 100 bp DNALadder, Fermentas, St. Leon-Rot).

#### **4.3.6. Isolation of DNA from agarose gels**

After gel electrophoresis, DNA fragments were isolated by excising the respective piece of agarose using a scalpel and an UV-transilluminator. DNA was extracted from the agarose using the PCR and gel extraction kit (Qiagen) or the GeneJET™ gel extraction kit (Fermentas) according to the manufacturer's instructions.

**4.3.7. Ligation of DNA fragments**

T4-DNA ligase reaction buffer: 50 mM Tris-HCl (pH 7.5)  
10 mM MgCl<sub>2</sub>  
10 mM dithiothreitol  
1 mM ATP

Amounts of isolated DNA fragments (insert) and linearized vectors were estimated on an ethidium bromide-containing agarose gel. The reaction sample with a total volume of 20 µl usually contained 50 ng of vector DNA, the three-fold molar amount of insert (in comparison to vector DNA) and 1 µl of T4 DNA ligase (supplied by Markus Hermann, AG Stemmann). The reaction mix was incubated for 1 h at RT in recommended amounts of T4-DNA ligase reaction buffer. Ligation mix was directly used for transformation (see 4.2.5).

**4.3.8. Sequencing of DNA**

Sequencing of plasmid DNA performed with the DYEnamic ET Terminator Cycle Sequencing Premix kit according to the manufacturer's instructions (GE Healthcare). One sample usually contained 700 ng of plasmid DNA and 20 pmol of primer filled with water to a total volume of 7 µl. DNA sequencing was then carried out by the core facility MPI for biochemistry (Martinsried) with an Abi-Prism 377 sequencer (Perkin Elmer) or by an external commercial provider (SeqLab, Göttingen).

**4.3.9. Polymerase chain reaction (PCR)**

PCRs were usually performed in a total volume of 50 µl with 1-10 ng of plasmid DNA or 0,1 µl of *Xenopus laevis* cDNA library (AG Stemmann), respectively, 0.2 µl of the respective forward and reverse oligonucleotide primers (10 µM each), 1 µl deoxynucleotide mix (10 mM each, New England Biolabs, USA) and 1 U of Phusion DNA polymerase (Finnzymes, Finland) or 1 µl Pfu DNA polymerase (gift from Markus Helfrich, Jennewein Biotechnologie GmbH, Rheinbreitbach), respectively, in the corresponding PCR buffer (5x Phusion HF or GC buffer, Finnzymes; 10xPfu buffer, Fermentas). Amplification was carried out in a TC-512 temperature cycler (Techne, Burlington, USA). The reaction profile was adjusted according to quantity and quality of template DNA, the length and G/C

content of the oligonucleotides, the length of the amplified sequences and the applied DNA polymerase.

#### **4.3.10. Site directed mutagenesis of DNA**

Site directed mutagenesis was performed in a PCR-based approach using two reverse complementary DNA oligonucleotides, each harboring the desired mutation(s). In two separate PCR reactions each DNA oligonucleotide was used to create an upstream and a downstream fragment, respectively. The outer primers were designed to terminate at useful restriction sites. After gel purification, the two products were combined and fused in a single PCR reaction with the two outer primers. The resulting fragments were restriction cloned into the desired vector.

### **4.4. Cell biological methods**

#### **4.4.1. Mammalian cell lines**

- Hek293T: human embryonic kidney cell line transformed with SV40 large T antigen
- HelaL: human cervix epithelial adenocarcinoma transformed by human-pathogene Papilloma virus, subclone L
- HCT116: human colon cancer cells, TGF1- $\beta$  positive, originated from a hereditary nonpolyposis colorectal cancer patient
- Hela FlpIn: human cervix epithelial cells modified by stable integration of a pFRT/*lacZeo* plasmid (Invitrogen) carrying the FRT recognition site for transgene integration by Flp-recombinase (mediates zeocin resistance), and stable integration of a pcDNA6/TR plasmid (Invitrogen; modified by replacing the *blastR* gene with a *puroR* gene; mediates puromycin resistance), for constitutive expression of the Tet-repressor. This host cell line was kindly provided by Thomas U. Mayer (University of Konstanz)
- Hek FlpIn: Flp-In™ T-REx™ 293 Cell Line. Source: Invitrogen Inc, USA

**4.4.2. Cultivation of mammalian cells**

1xPBS: 137 mM NaCl  
2.7 mM KCl  
10 mM Na<sub>2</sub>HPO<sub>4</sub>  
2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, PAA, Austria). Media was supplemented with 10% heat inactivated (40°C, 10 min) fetal bovine serum (Biochrom, Berlin; Sigma-Aldrich, Taufkirchen), 100 units/ml penicillin and 0.1 mg/ml streptomycin (PAA). This medium was further supplemented with 4 µg/ml puromycin (Enzo Life Sciences, Plymouth Meeting, PA, USA) and 62.5 µg/ml zeocin (Invitrogen) for culturing Hela FlpIn cells. Monolayer cultures were grown in cell culture dishes (Greiner Bio-One, Kremsmünster, Austria) at 37°C in a restricted 5% CO<sub>2</sub> atmosphere and were split at a ratio of 1:4 to 1:8 twice a week. To split cells, medium was removed, cells were washed once with 1xPBS and subsequently incubated with 0.5-2 ml Trypsin/EDTA solution (PAA) at 37°C for 3 min (Hek293T), 10 min (HCT116) or 15 min (HeLa/HeLa FlpIn). By repeated pipetting in fresh medium, cells were further detached from each other as well as from the culture dish. Subsequently, the cell suspension was diluted in medium and distributed on fresh culture dishes. Cell concentrations of suspensions were determined with a Coulter Counter or a Vi-Cell counter (Beckman Coulter, Krefeld).

**4.4.3. Storage of mammalian cells**

Freezing medium: 10% DMSO  
90% fetal bovine serum

Cells were harvested at 60-80% confluence by trypsination as described in chapter 4.4.2), resuspended in freezing medium and pipetted in cryo vials (SARSTEDT, Nümbrecht). The cell suspension was cooled to -80°C in an insulated device containing isopropanol. For long-term storage, cryo vials were stored in a liquid nitrogen tank. For thawing, cryo vials were quickly removed from the liquid nitrogen freezer and placed into a 37°C water bath. To remove freezing medium, tubes were briefly centrifuged

(300 g, 3 min) and the supernatant was discarded. The cells were washed once in 1xPBS to remove residual amounts of DMSO. After another centrifugation round, the cell pellet was resuspended in DMEM and transferred to an appropriate cell culture dish containing DMEM.

#### **4.4.4. Transfection of 293T cells**

2xHBS (50 ml):   800 mg NaCl  
                          37 mg KCl  
                          10.65 mg Na<sub>2</sub>HPO<sub>4</sub>  
                          100 mg glucose  
                          500 mg HEPES pH 7.05 adjusted with NaOH, sterile filtered (0.2 µm pore size)

Hek293T cells were transfected by the calcium phosphate method.  $2.5 \times 10^6$  cells per cell culture dish (Ø 100 mm) were spread and grown overnight. One hour prior to transfection, old media was removed and fresh media was added. Shortly before transfection, chloroquine was added to the medium to a final concentration of 30 µM. For one transfection mix, 5-20 µg of plasmid DNA was mixed first with 680 µl water and then with 99.2 µl sterile 2 M CaCl<sub>2</sub>. 800 µl of 2xHBS solution was slowly added in small drops while vortexing. The transfection mix was immediately added to the cells by careful dripping onto the entire surface of the culture dish. 8-12 h later or overnight, medium was exchanged. In most cases during this study, transfection was performed between two thymidine treatments (see 4.4.7) of a double thymidine-nocodazole block. Cells were harvested by rinsing media over the cells and pelleted by centrifugation (3 min, 300 g). After a washing step with 1xPBS, the cells were either processed immediately or shock-frozen in liquid nitrogen and stored at -80°C for later use.

#### **4.4.5. Transfection of HeLa cells**

HeLa cells were either transfected using the cationic lipid reagent Lipofectamine 2000 (Invitrogen), Lipofectamin RNAiMax™ (Invitrogen) or by polyethylenimine (PEI, linear, MW 25,000, Polysciences, Inc., Warrington, PA, USA), a cationic polymer.

In the case of Lipofectamine 2000 a 1:2 ratio of DNA (µg):Lipofectamine (µl) was used.

Cells were seeded in six-well plates in 2 ml DMEM medium supplemented with fetal bovine serum without antibiotics at a confluence of about 60%. Transfection mixes were prepared in two steps. First, 1.6 µg of DNA were diluted in 100 µl OptiMEM (Invitrogen). In a separate tube, 3.2 µl of Lipofectamine 2000 were mixed well with 100 µl of OptiMEM. After 5 min incubation at RT, the DNA solution was pipetted to the Lipofectamine solution and mixed well. Following another incubation for at least 20 min at RT, the transfection mix was added to the cells. To limit cytotoxicity, medium was changed after 4-6 h, this time using medium with antibiotics.

For transfection of dsRNAs for RNAi experiments, 200 µl OptiMem was supplemented with 3 µl RNAiMax™. Another tube containing 200 µl of OptiMem was supplemented with 100 pM of dsRNA. After briefly mixing and incubating both tubes for 5 min at RT, the solutions were combined, mixed and further incubated for 20 min. This transfection mix was then pipetted to cells of a well of a six-well plate.

For PEI transfections, the ratio of DNA (µg):PEI (µl of 1 µg/µl stock solution) was 1:4. Cells were seeded at 10<sup>6</sup> cells per cell culture dish (Ø 100 mm). 1 µg/ml plasmid DNA was incubated in 1 ml DMEM medium without fetal bovine serum or antibiotics for 2 min at RT. After PEI addition, the transfection mix was vortexed, incubated at RT for 10 min and added to the cells for 48 h.

#### **4.4.6. Generation of stable cell lines**

HeLa FlpIn cell lines with stable, inducible transgenic expression were generated using the HeLa FlpIn host cell line (provided by Thomas Mayer, Konstanz; see chapter 4.4.1). Briefly, plasmid DNA (Flag<sub>3</sub> epitope tagged Pin1 constructs or Myc<sub>6</sub> epitope tagged Cdc20 constructs in pcDNA5-FRT-TO vector background) and Flp integrase expression plasmid DNA (pOG44, Invitrogen) were transfected at a ratio of 1:10 using Lipofectamine 2000 (Invitrogen/Fisher Scientific) or polyethylenimine (PEI, linear, MW 25,000, Polysciences, Inc., Warrington, PA, USA; see chapter 4.4.5). 24 h after transfection, cells were split to a lower density. Cells were further incubated for 24 h followed by addition of 400 µg/ml hygromycin (PAA). During a period of two weeks medium was exchanged several times. Once hygromycin resistant colonies were large enough to see by eye, they were re-plated. The clones were further expanded to six-well plates



(Greiner Bio-One) and tested for inducible expression of the transgene.

#### **4.4.7. Synchronization of mammalian cells**

Synchronization of cells at the G1/S boundary of the cell cycle was performed by adding 2 mM thymidine (Sigma-Aldrich) to the culture medium for 18 h. Cells were then released from the block by washing twice with 1xPBS followed by a 15 min incubation step in the cell culture incubator and medium exchange. 2 mM thymidine was added to the cells 9 h after release from the first thymidine block. 17 h later cells were released as described above. Synchronization of cells in prometaphase of mitosis was performed by adding 200 ng/ml nocodazole (if not indicated otherwise) 3 hours after release from the 2<sup>nd</sup> thymidin block. For certain immunoprecipitation experiments, cells were synchronized in mitosis by treating them with a single thymidine block for 24 h followed by washing with the same procedure described above and adding nocodazole 3 hours after release. Nocodazole treatment was carried out for 14 h if not indicated otherwise. In some experiments cells were treated with the proteasome inhibitor MG-132 (Enzo Lifescience), the Pin1 inhibitor dipentamethylene thiuram monosulfide (GP Biochemicals, OH, USA), the Pin1 inhibitor epigallocatechin gallate (Sigma-Aldrich) or the protein biosynthesis inhibitor cycloheximid (Sigma-Aldrich) at the indicated time points using the indicated concentrations

#### **4.4.8. Quantitative analysis of cell cycle stages**

To identify the distinct cell cycle stages, cells were trypsinated from the cell culture dish, pelleted (300 g, 3 min) and transferred to a 15 or 50 ml Falcon (Greiner Bio-One) tube. After pelleting, cells were washed once with 1xPBS to remove residual medium. Cells were subsequently fixed with -20°C cold 70% ethanol o.n. at 4°C, washed twice with 1xPBS and passed through a 35 µm nylon mesh cup of a FACS tube (BD Biosciences). DNA staining was performed by incubating the cells with a 69 µM propidium iodide solution (in 38 mM tri-sodium citrate, Sigma-Aldrich) supplemented with 100 µg/ml RNase A (Qiagen) for 1 h at 37°C. DNA content was determined using a Beckman Coulter Cytomics FC 500 flow cytometry device and the corresponding PC software CXP Analysis (Beckman).

**4.4.9. Immunofluorescence staining of cells**

Fixation buffer:	1xPBS 0.3% Triton X-100 3.7% formaldehyd	Permeabilization buffer:	1xPBS 0.5% Triton X-100
Washing buffer:	1xPBS 0.1% Triton X-100	Blocking buffer:	1xPBS 0,1% Triton X-100 3% BSA
Stopping buffer:	1xPBS 100 mM glycine	Mounting medium:	2,33 % diaza-bizyklo-(2,2,2)-oktan (w/v) 20 mM Tris/HCl, pH 8,0 78 % glycerol (v/v)

For immunofluorescence staining, cells grown on coverslips were washed with 1xPBS in 6-well or 24-wells culture plates. The cells were then fixed for 8 min in fixing buffer followed by two washing steps with stopping buffer and one wash step with 1xPBS. Samples were permeabilized by incubation in permeabilization buffer for 5 min. After washing once with 1xPBS, samples were incubated in blocking buffer for 45 min at RT or o.n. at 4°C. Coverslips were transferred onto parafilm inside a wet chamber. Staining was performed by incubation with an appropriate dilution of primary antibodies in blocking buffer for 90 min followed by 3 washing steps with washing buffer. After incubation with a 1:750 dilution of fluorescently labeled secondary antibodies (Invitrogen, dianova and Bethyl) for 45 min, samples were incubated in washing buffer containing 2 µg/ml Hoechst 33342 for 10 min and washed five times. Coverslips were finally mounted with 3 µl mounting medium and placed on a glass slide. Preparation was finished by sealing the edges of the cover slips with transparent nail polish (Rossmann). Images were acquired using an Axiovert upright microscope (Carl Zeiss MicroImaging, Jena).

**4.4.10. Determination of the mitotic index by flow cytometry**

Cells were harvested by trypsination and the resuspended cells were pooled with the material derived from culture supernatant and 1xPBS wash. Cells were fixed by addition of 1/20 volume of a 35 % formaldehyd solution for 15 min at RT. The cells were washed twice with 1xPBS containing 100 mM glycine and then permeabilized with methanol precooled to -20°C for 20 min on ice. The cells were then washed once with 1xPBS/0.2% BSA and once with 1xPBS/2% BSA. After 30 min blocking in 1xPBS/2% BSA, the supernatant was removed completely and the cells were incubated for 45 min with anti-phospho histone H3 antibody (1:100 dilution in 1xPBS/2% BSA, Sigma Aldrich). After two washing steps with 1xPBS/0.2% BSA and 1xPBS/2% BSA, respectively, the cells were stained with Cy3 labeled goat anti-mouse antibody (1:100 dilution in 1xPBS/2% BSA, Invitrogen). The cells were then washed once with 1xPBS/0.2% BSA and resuspended in 1xPBS. The cell suspension was analyzed with the Beckman Coulter Cytomics FC 500 flow cytometry device using the FL-2 channel.

**4.4.11. Life-cell analysis**

Live cell imaging was carried out on a DMI 6000 inverted microscope (Leica Microsystems, Wetzlar) equipped with a digital camera (Leica Microsystems, Wetzlar) and with an HCX PL FLUOTAR L 20x/0.4 objective (Leica Microsystems, Wetzlar). Cell culture conditions were provided by a temperature controlled chamber maintained at 37°C and a small lid covering the sample for application of a humidified 5% CO<sub>2</sub> atmosphere (Pecon, Erbach). Pictures of cells were taken after a mitotic shake-off on standard 12 or 24 well plate (Greiner-One). A typical movie was acquired with the phase contrast method at a frame rate of 15 min.

**4.4.12. Preparation of sperm nuclei from *X. laevis***

2xXN buffer:	100 mM HEPES KOH pH 7.0	XN buffer	3% BSA
	500 mM sucrose	+ 3% BSA:	50% 2xXN
	150 mM NaCl		filled to 100% with
	1.0 mM spermidine		H <sub>2</sub> O
	0.3 mM spermine		

XN/ 50% glycerol:    50 % 2xXN  
                             50 % v/v glycerol

Five days prior to isolation of the testis, two *X. laevis* male frogs were injected with 25 U gonadotropin from pregnant mare serum (Sigma-Aldrich) into the dorsal lymph sac using a 27-gauge needle (B. Braun, Melsungen). One day before isolation, frogs were injected with 50 U chorionic gonadotropin (Sigma-Aldrich). The next day, the frogs were sacrificed by incubating them in water containing 0.05% benzocaine for 15-20 min. The testes were isolated and transferred into a petri dish containing 1xMMR buffer (see 4.4.13). By using fine forceps and a binocular, all remaining blood vessels and fat tissue were removed. Testes were transferred to a fresh petri dish containing 1xXN buffer. Sperm cells were isolated by scratching the testes with forceps. The suspension was squeezed through a 100 µm cell strainer (BD Biosciences) and transferred to a 15 ml Falcon tube (Greiner Bio-One). After centrifugation (3200 g, 5 min, 4°C), the supernatant was discarded, 2 ml XN buffer was added and the sperm cells, visible as a white pellet, were carefully transferred into a fresh tube without dispersing the remaining blood cells, which were situated at the bottom of the tube. This step was repeated twice and the sperm cells were resuspended in 2 ml XN buffer. To demembranate the sperm cells, the suspension was incubated with 400 µl 2 mg/ml L-α-lysophosphatidyl-choline (from egg yolk, Sigma-Aldrich) in 1xXN buffer for 1 h at RT. Subsequently, the reaction was quenched by adding 9.5 ml 1xXN + 3% BSA. The sperm nuclei were washed once with 1xXN, once with 1xXN + 3% BSA and again with 1xXN and resuspended in an appropriate volume of 1xXN/50% glycerol. Aliquots were frozen at -80°C. The concentration of sperm nuclei derived from the preparation was estimated using a haemocytometer.

#### 4.4.13. Preparation of CSF-extract

<i>MMR (25x):</i>	2.5 M NaCl 50 mM KCl 25 mM MgCl <sub>2</sub> 50 mM CaCl <sub>2</sub> 2.5 mM EDTA/NaOH (pH 8.0) 125 mM HEPES/NaOH (pH 7.8) pH 7.8, adjusted with NaOH	<i>XB-salts (20x):</i>	2 M KCl 2 mM CaCl <sub>2</sub> 20 mM MgCl <sub>2</sub>
<i>CSF-XB:</i>	100 mM KCl 0.1 mM CaCl <sub>2</sub> 2 mM MgCl <sub>2</sub> 10 mM HEPES/KOH (pH 7.7) 50 mM sucrose 5 mM EGTA/KOH (pH 8.0) pH 7.7, adjusted with KOH	<i>XB:</i>	100 mM KCl 0.1 mM CaCl <sub>2</sub> 2 mM MgCl <sub>2</sub> 10 mM HEPES/KOH (pH 7.7) 50 mM sucrose pH 7.7, adjusted with KOH
<i>Dejelling solution:</i>	2% (w/v) cysteine (free base) 0.5x XB-salts pH 7.8, adjusted with KOH	<i>Cytochalasin B:</i>	10 mg/ml in DMSO (1000x)
<i>Sperm dilution buffer:</i>	5 mM HEPES/KOH (pH 7.7) 100 mM KCl 150 mM sucrose 1 mM MgCl <sub>2</sub>	<i>Ca<sup>2+</sup> (25x):</i>	15 mM CaCl <sub>2</sub> in sperm dilution buffer

Unfertilized eggs laid by *X. laevis* are arrested in prometaphase II of meiosis. Release into interphase is triggered by Ca<sup>2+</sup>-ions. Therefore, all glassware used for extract preparation was rinsed twice with ddH<sub>2</sub>O to remove contaminating residual calcium ions. Work with frogs and frog eggs was performed at 18°C and all buffers and equipment

were stored at that restrictive temperature.

To induce egg laying, female frogs were injected with 0.7-1 ml chorionic gonadotropin (1000 U/ml, Sigma-Aldrich) into the dorsal lymph sac using a 27-gauge needle (B. Braun, Melsungen). 8 h after injection, frogs were individually transferred into vessels containing 1,5-2 l 1x MMR buffer. 20-24 h after injection, laid eggs were collected, washed twice with 1x MMR and transferred into a bottomed glass dish. "Bad" looking eggs (those with abnormal morphology) were removed using a Pasteur pipette. The jelly coats of the eggs were removed by incubating them in dejellying solution for 5-10 min. The eggs were subsequently washed 6 times with CSF-XB to remove residual amounts of cysteine. Again, eggs with abnormal morphology were removed. The remaining eggs were transferred into a 12 ml centrifuge tube containing 01 ml CSF-XB and 10 µl cytochalasin B solution. The tubes were centrifuged in a JS13.1 rotor (Beckman) for 1 min at 200 g and 1 min at 600 g. After removing the exterior buffer from the eggs, the tubes were centrifuged for 10 min at 13,000 g. After this so called crushing spin, the tube was punctured with an 18-gauge needle at the lower end of the middle layer. This cytoplasmatic fraction was pulled out and transferred to a 1,5 ml reaction tube. Cytochalasin B was added to the extract at a final concentration of 10 µg/ml. From now on, extracts were stored on ice until use.

Prior to all extract-based experiments, the quality of the extract was assessed. Therefore, a small portion of the CSF extract was transferred to a reaction tube and supplemented with low amounts of *X. laevis* sperm for several minutes. A part of this mixture was further transferred to a reaction tube containing CaCl<sub>2</sub> (0.6 mM final concentration). After 30 min incubation at 30°C it was tested, whether the extract without CaCl<sub>2</sub> was properly arrested in prometaphase (indicated by condensed chromatin) and the CaCl<sub>2</sub>-containing extract was released into interphase (indicated by round nuclei).

For several experiments, extracts were immunodepleted with xPin1- or unspecific IgG. For that purpose 40 µl magnetic protein G Dynal beads (Invitrogen, USA) were coupled with 12 µg xPin1 antibody or unspecific rabbit IgG (Bethyl) according to the manufacturer's instructions, and then washed three times with XB buffer. 50 µl extract was mixed with the beads and immunodepleted for 45 min at 18°C. Using a magnet (Invitrogen, USA), the depleted extract was separated from the magnetic beads and transferred

to a fresh tube.

In initial experiments, the role of Pin1 in the spindle assembly checkpoint (SAC) was investigated using SAC-activated extracts of *Xenopus* eggs. The SAC was established by adding 14,000  $\mu\text{g/ml}$  sperm nuclei (final concentration) and 50  $\mu\text{g/ml}$  nocodazole to freshly prepared CSF extracts.

**4.4.14. Re-Isolation of sperm nuclei from CSF-extract**

OMD:	10 mM Hepes	OMF:	10 mM Hepes
	70 mM KCl		70 mM KCl
	0,5mM $\text{MgCl}_2$		0,5mM $\text{MgCl}_2$
	1 mM EGTA		1 mM EGTA
	0,25 M sucrose		10% glycerol
	pH 7.7 adjusted with KOH		0,1% Triton X-100
			(2,7% formaldehyde)
			pH 7.7 adjusted with KOH
OMC:	10 mM Hepes		
	70 mM KCl		
	0,5mM $\text{MgCl}_2$		
	1 mM EGTA		
	40% glycerol		
	pH 7.7 adjusted with KOH		

For immunofluorescence analysis of sperm nuclei from *X. laevis* egg extracts, a chromatin re-isolation was performed. After incubation of the sperm nuclei, the extracts were diluted in a ratio of 1:4 with OMD buffer. After incubation at RT for 15 min, extracts were then diluted with OMF buffer in a ratio of 1:4 and gently mixed for 5 min. The extracts were applied on top of a cushion (containing of 3,5 ml OMC buffer) and centrifuged on coverslips (20 min, 11,000 g, 4°C) using a JS 13.1 swing-out rotor (Beckman) and Corex centrifuge tubes with the appropriate adaptors for the coverslips (Corex,

USA). Afterwards, coverslips were washed several times with 1xPBS containing 0,1% Triton-X 100 and further processed for immunofluorescence staining.

#### **4.5. Proteinbiochemical methods**

##### **4.5.1. Measurement of Pin1 enzymatic activity**

The measurement of the *in vitro* catalytic activity of bacterially expressed xPin1 variants was performed in the laboratory of Prof. Franz-Xaver Schmid with essential support from Dr. Gabriel Zoldak using a peptide-based assay. The Pin1 pseudo-substrate Abz-AEPF-pNA (Aminobenzyol-Alanine-Glutamate-Proline-Phenyl-alanine-p-Nitroanilide) was used for measurement of the enzymatic activity. This peptide contains a fluorophore (Abz) and a quencher (pNA) and is present in the *trans*- and *cis*-conformation in equal when diluted in trifluoro acetic acid (TFA). In its solvent, the fluorescence of the peptide in its *cis*-conformation is suppressed by the close proximity of fluorophore and quencher. Upon addition of the peptide to an aqueous solution the conformation is switched within minutes from the *cis*- to the *trans*-form. Thus, the fluorophore and the quencher are not in close proximity anymore and the peptide gains fluorescence activity. Upon addition of a prolyl isomerase (in this case Pin1) this conformational switch can be dramatically accelerated. Fluorescence dynamics were measured using a fluorescence spectrophotometer.

A glass cuvette containing 1198.8 µl XB buffer, 1,2 µl BSA (from a 1 mM stock solution) and 12 µl bacterially expressed xPin1 (from a 1 µM solution) was placed in the measurement device of the fluorescence spectrometer and equipped with a magnetic stir bar. Shortly after, 5 µl of the peptide (from a 1 mM stock) was added to the solution and fluorescence of the peptide was measured using the settings listed above. This procedure was repeated for different concentrations of bacterially expressed xPin1, different xPin1 variants and to test the Pin1 inhibitor dipentamethylene thiuram monosulfide for its ability to inhibit Pin1 catalytic *in vitro* activity.



**4.5.2. SDS-polyacrylamid gel electrophoresis**

<i>Laemmli running</i>	25 mM Tris	<i>4X Sample buffer:</i>	250 mM Tris-HCl (pH 6.8)
<i>buffer:</i>	192 mM glycine		40% glycerol
	3.5 mM SDS		8% (w/v) SDS
			2 M $\beta$ -mercaptoethanol
			0,04% (w/v) bromphenol blue

For the separation of proteins under denaturing conditions, SDS-PAGE was performed using commercially available "SERVAGel™ Neutral pH 7.4" gradient gels (SERVA) or self made gradient gels provided by technical assistants. Prior to loading, protein samples were mixed with 4x sample buffer and denatured at 95°C for 5-15 min. In some cases cells or CSF extract were directly boiled in 1xsample buffer. As a molecular weight standard, PageRuler Prestained Protein Ladder (Fermentas) was used. Electrophoresis was carried out at a constant voltage of 140 V in Laemmli running buffer for 90 min using electrophoresis chambers from Invitrogen or Hoefer.

**4.5.3. Immunoblotting**

<i>Towbin buffer (1x):</i>	25 mM Tris	<i>TBSW:</i>	25 mM Tris (pH 7.5)
	192 mM glycine		137 mM NaCl
	20% methanol		2.6 mM KCl
			0.05% Tween-20
			(blocking solution: TBSW + 1 % BSA)

Proteins were separated by SDS-PAGE as described in section 4.5.2. Afterwards, proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon P, Millipore; Fluorobind, SERVA) using Towbin buffer and a "semi-dry" blotting apparatus (Peqlab, Biorad or Hoefer). Prior to blotting, the PVDF-membrane was shortly soaked with methanol and washed several times with ddH<sub>2</sub>O. Supportingly, extra thick blot papers (Biorad) were used to cover membrane and gel during the blotting process. Protein transfer was carried out at a constant voltage of 15 V for 90 min at RT. The membrane was blocked for unspecific binding using blocking

solution for 1 h at RT or overnight at 4 °C. Incubation of the membrane with the primary antibody diluted in blocking solution was done at RT for 1 h. The membrane was subsequently washed three times with TBSW for 5 min each. The appropriate horseradish peroxidase (HRP)-coupled secondary antibody was diluted in TBSW and added to the membrane for 60 min. Usually the antibody was applied using a concentration of 1:5000 (HRP coupled bovine anti-goat; bovine anti-sheep) or 1:50,000 (all other HRP coupled secondary antibodies). Afterwards, the membrane was washed three times with TBSW for 10 min each. Chemiluminescence detection was carried out using the protocol provided with the kit (ECL, GE Healthcare or HRP-Juice, PJK GmbH, Kleinblittersdorf) and CCD-based LAS-3000 or LAS-4000 camera systems (Fuji, Japan; now GE Healthcare, USA).

#### **4.5.4. Coomassie staining**

*Coomassie solution:*   80 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
                                  12 % phosphoric acid (v/v)  
                                  0.8 g Coomassie G250  
                                  781.2 ml ddH<sub>2</sub>O

For coomassie staining, gels were incubated in coomassie solution after SDS-PAGE for at least 2 h. To remove unspecific stain, gels were incubated in ddH<sub>2</sub>O overnight. For rapid destain, gels were incubated in boiling water for 10 min. For long-term storage, coomassie-stained gels were dried on Whatman blotting paper (GE Healthcare) in a slab gel dryer (GD2000, Hoefer).

#### **4.5.5. Autoradiography**

*Destain solution:*   45% methanol  
                                  45% ddH<sub>2</sub>O  
                                  10% acetic acid

After SDS-PAGE, the gel was incubated in destaining solution for 30 min at RT. To remove residual <sup>35</sup>S-methionine, the gel was incubated in ddH<sub>2</sub>O for 15 min. The gel was

dried using a gel dryer (GD2000, Hoefer) according to the manufacturer's instructions. Signal detection was performed using an FLA-7000 system (Fuji, Japan; now GE Healthcare, USA).

#### **4.5.6. *In vitro* transcription/translation (IVT)**

The "TNT Quick Coupled Transcription/Translation System" (Promega, Mannheim) was used for *in vitro* translation of radioactively labeled proteins. 20 µl "TNT Quick Master Mix", 1 µl plasmid DNA (500 ng/µl), 1 µl <sup>35</sup>S-methionine (Hartmann Analytic GmbH, Braunschweig) and 3 µl RNase free water were combined and incubated at 30°C for 90 min. Alternatively, the TnT® Coupled Wheat Germ Extract System (Promega, Mannheim) was used according to the manufacturer's instructions.

#### **4.5.7. Affinity purification of His<sub>6</sub>- or GST tagged proteins**

Lysis buffer I:	400 mM NaCl	Lysis buffer II:	400 mM NaCl
	1x PBS		1x PBS
	5 mM β-mercaptoethanol		5 mM DTT
	20 mM imidazole		
Elution buffer I	Lysis buffer I + 230 mM imidazole pH 7.5	Elution buffer II:	Lysis buffer II + 10 mM glutathione pH 7.5

For several purposes, proteins were recombinantly expressed in *E. coli*. Expression was performed using expression strains listed in chapter 4.2.6. After protein expression (see. 4.2.6), bacteria were harvested and resuspended in 10 ml of ice-cold lysis buffer per g pellet. Importantly, for His<sub>6</sub>-SUMO3-tagged proteins, lysis buffer I was used, for GST-tagged proteins resuspension was performed with lysis buffer II. Cells were lysed in a homogenizer (Avestin, Ottawa, Canada) by cycling the cell suspension for 5-10 min according to the manufacture's instruction. The lysate was cleared from debris by centrifugation in a JA-25.50 rotor (Beckman Coulter) at 40,000 g for 20 min (4°C). The supernatant was incubated with 250 µl Ni<sup>2+</sup>-NTA agarose (Qiagen, for 500 ml of expression culture) for His<sub>6</sub>-SUMO3-tagged proteins and 500 µl glutathione sepharose (GE

Healthcare) for GST-tagged proteins and incubated on a turning wheel for 1-3 h at 4°C. Beads were washed 5-6 times with the appropriate lysis buffer and then incubated with elution buffer I (for His<sub>6</sub>-SUMO3-tagged proteins) for 5-10 min or elution buffer II (for GST-tagged proteins) for 30 min at 4°C and transferred to a 5 ml polycarbonate column (Bio-Rad). The flow-through containing the eluted proteins was collected in a fresh tube. After elution, His<sub>6</sub>-SUMO3-tagged proteins were incubated with 10 µg His<sub>6</sub>-Senp2-protease (Core-Facility, MPI) for 30 min at 4°C followed by a second round of incubation with Ni<sup>2+</sup>-NTA agarose to remove the His<sub>6</sub>-SUMO3-tag and the His<sub>6</sub>-Senp2 protease from the eluate. The suspension was again transferred to a column and the flow-through containing the purified protein was collected. Finally, the proteins were dialyzed overnight against appropriate buffers at 4°C using “slide a lyzer” membranes (Thermo Scientific). During the dialysis process, the buffer was exchanged several times. For some purposes the protein was further concentrated using Spin-X UF 2 ml centrifugal concentrators (Milipore). For some pulldown experiments, protein-coupled agarose or sepharose beads were resuspended in 1xPBS containing 0,02% sodium azide after the washing procedure and stored at 4 °C prior to use.

#### **4.5.8. Purification of antibodies out of serum**

For the generation of polyclonal antibodies against *X. laevis* Pin1, recombinant full-length GST-tagged xPin1 was expressed in *E. coli* Rosetta and purified as described in chapter 4.5.7. 1 mg of antigen in 1xPBS buffer was thoroughly mixed with TiterMax™ Gold Adjuvant (Sigma) and used to immunize a rabbit at the Immunization Facility of the MPI of Biochemistry. 6 weeks later, the rabbit was bled to death and blood was centrifuged (5000 g, 30 min, 4 °C) to obtain the serum. The serum was further passed through a 0.2 µm filter. Antibodies raised against the antigen were purified by pumping the obtained rabbit serum over a HiTrap N-hydroxy-succinimide (NHS)-activated column (GE Healthcare) coupled with bacterially expressed untagged xPin1 according to the instructions of the manufacturer at a rate of 0.5 ml/min. Coupling was performed with bacterially expressed full-length xPin1 using the pET28M-SUMO<sub>3</sub>-FA vector. After a washing step with 1xPBS and 1 mM Tris-HCl (pH 6.8), antibodies were eluted from the column with 100 mM glycine/100 mM NaCl (pH 2.5). The eluted fractions were rapidly

neutralized using 1/10 volume 1 M Tris pH 9.0. Fractions containing antibodies were identified by SDS-PAGE and Coomassie staining described in sections 4.5.2 and 4.5.4. Peak fractions were pooled, dialyzed against 1xPBS/50% glycerol and stored at -20°C.

#### **4.5.9. Co-Immuno-Precipitation (Co-I.P.) and pulldown experiments**

Lysis buffer 2    20 mM Tris-HCl (pH7.7)  
(LP2):            100 mM NaCl  
                      10 mM NaF  
                      20 mM  $\beta$ -glycerophosphate  
                      5 mM MgCl<sub>2</sub>  
                      0.1% Triton X-100  
                      5% glycerol  
                      1 mM EDTA  
                      1 tablet Roche Complete Protease Inhibitor per 50 ml buffer

Cells were harvested or rinsed from confluent 100 mm cell culture dishes. After centrifugation for 3 min at 300 g and RT, cells were washed once with 1xPBS and centrifuged again. From here on all steps were performed on ice. Cells were processed immediately or snap-frozen and stored at -80°C for later use. The cell pellets were resuspended in 500  $\mu$ l LP2 buffer, dounce-homogenized (using a glass dounce homogenizer and a tight pestle, Wheaton, USA) and left on ice for 10 min. Lysates were centrifuged at 35,000 rpm using a TLA-55 rotor for 30 min at 4 °C and the supernatant was transferred to a new tube. An input sample of 45  $\mu$ l was extracted and mixed with 15  $\mu$ l 4xSDS-PAGE sample buffer. Concomitantly, the appropriate affinity matrix was equilibrated by washing twice with LP2 buffer. For immunoprecipitation of endogenous proteins, 5  $\mu$ l ProteinG or ProteinA sepharose (GE Healthcare) were coupled with 10  $\mu$ g antibodies according to the manufacturer's instructions. For co-I.P. of Flag-tagged proteins, 5  $\mu$ l anti-FLAG M2-Agarose from mouse (Sigma) was used. Subsequently, the supernatants were incubated with the equilibrated beads at 4°C for 2-4 h (FLAG co-I.P.) or o.n. (IP of endogenous proteins). Beads were then washed five times with LP2 buffer if not indicated otherwise. The proteins were diluted by denaturing the beads in 1xSDS-

sample buffer at 95°C for 15 min. Alternatively, the beads were incubated in 1xSDS sample buffer without DTT and  $\beta$ -mercaptoethanol at 75 °C for 15 min. After transferring the protein containing eluate into fresh tubes using MoBiCols microcolumns (Morbitec, Göttingen),  $\beta$ -mercaptoethanol was added at a final concentration of 500 mM and the eluate was denatured for 5 min at 95°C.

In some pulldown approaches, GST-tagged proteins coupled to glutathione sepharose beads were added to LP2 lysates. Incubation was performed at 4 °C for 2 h using 10  $\mu$ l beads for each sample. The sepharose beads were washed 5 times with LP2 buffer if not indicated otherwise and directly boiled in 1xSDS sample buffer.

#### ***4.5.10. Cdk1/MAPK kinase and lambda phosphatase treatment***

For one experiment, GST fusion proteins coupled to glutathione sepharose were incubated with 100 U lambda phosphatase using the appropriate buffer concentrate and  $\text{MnCl}_2$  according to the manufacturer's instructions (New England Biolabs, Frankfurt a. M.). In another experiment, His<sub>6</sub>-SUMO-fusion proteins coupled to Ni<sup>2+</sup> NTA agarose were incubated with 10 U Cdk1 kinase and 10 U MAPK kinase (New England Biolabs) using the appropriate buffer, 1 mM ATP and 300  $\mu\text{Ci}/\mu\text{mol}$  [ $\gamma$ -<sup>33</sup>]ATP. Samples were incubated for 30 min at 30°C.

## 5. ABBREVIATIONS

aa	amino acid(s)
Ab	Antibody
APC/C	anaphase promoting complex/cyclosome
APOBEC	apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like
AR	Autoradiography
ATP	adenosine 5'-triphosphate
Bcl	B-cell lymphoma
BSA	bovine serum albumin
Bub	budding uninhibited by benzimidazole
Cdc	cell division cycle
Cdk	cyclin dependent kinase
Cenp	Centromere protein
Cep	Centrosomal protein
CHX	cycloheximid
CM	Coomassie
CMV	cyto megalovirus
CSF	cytostatic factor
Da	Dalton
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide
DTM	Dipentamethylene thiuram monosulfide
DTT	dithiotreitol
<i>E. coli</i>	escherichia coli
EDTA	ethylenediamine tetraacetic acid
EGCG	Epigallocatechin-gallate
EGTA	ethylen glycol tetraacetic acid
Emi	early mitotic inhibitor
Esp	extra spindle pole
FL3Lin	fluorescence parameters 3 linear
FLAG	epitope tag (aa sequence: DYKDDDDK)
g	gram or gravitational constant (9.81 m/sec)
GST	glutathion-S-transferase
HBS	HEPES buffered saline
Hec	highly expressed in cancer
HEPES	4-(2-hydroxyethyl)-1piperazineethansulfonic acid
HI	Human immunodeficiency
IF	immuno fluorescence
IgG	immunoglobulin G
IP	immunoprecipitation

IVT	<i>in vitro</i> transcription/translation
KNL	kinetochore-null
LB	Luria-Bertani
Mad	mitotic arrest deficient
MAPK	Mitogen-activated protein kinase
MCC	mitotic checkpoint complex
Mcm	mini-chromosome maintenance
MEF	mouse embryonic fibroblasts
MMR	Marc's modified Ringer
Mnd	meiotic nuclear divisions
MTOC	Microtubule organizing center
Myc	myelocytomatosis oncogene
NB	non binding
Ndc	nuclear division cycle
NF- $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B
NHS	N-hydroxysuccinimid
Noc	nocodazole
NTA	nitro tri-acetic acid
o/n	over night
OD	optical density
ORF	open reading frame
p.a.	<i>pro analysi</i>
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	pulldown
PEI	polyethylenimine
pH3	phosphorylated histone H3
Pin	peptidylprolyl cis/trans isomerase, NIMA-interacting
PKA	protein kinase A
Plk	polo like kinase
PP	Protein Phosphatase
PPiase	peptidyl-prolyl cis/trans isomerase
PVDF	Polyvinylidene fluoride
Rad	radiation sensitive
RNAse	ribonuclease
rpm	revolutions per minute
S	Sievert
S	Serine
SA	stromal antigen
SAC	spindle assembly checkpoint
SAP	shrimp alkaline phosphatase
Scc	sister chromatid cohesion
SDS	sodium dodecylsulfate
Sgo	shugoshin
Smc	structural maintenance of chromosomes
SUMO	small Ubiquitin-like Modifier



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TBS	Tris buffered saline (TBS-w: TBS with 0.1% Tween-20)
TEMED	N,N,N',N'-tetramethylethylenediamine
TEV	Tobacco Etch Virus
Tris	tris(hydroxymethyl)aminomethane
Ub	Ubiquitin
UbcH	ubiquitin
Ubi	Ubiquitin
v/v	volume per volume
w/o	without
w/v	weight per volume
WB	Western blotting
WCE	whole cell extract
WT	wildtype
<i>X. laevis</i>	<i>Xenopus laevis</i>
XB	extract buffer

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## 7. LEBENS LAUF

### Persönliche Daten

Name: Andreas Brown  
Geburtsdatum: 09.08.1982  
Geburtsort: Starnberg  
Adresse: Gottfried-Semper-Weg 36, 95444 Bayreuth

### Schulbildung

1989-1993 Grundschule Holzkirchen  
1993-2002 Gymnasium Oberhaching  
Abschluss mit Abitur im Mai 2002

### Studium

2002 - 2008 Ludwig-Maximilians-Universität München  
Studium der Biologie  
Abschluss mit Diplom im Januar 2008  
  
2007 - 2008 Diplomarbeit in der Arbeitsgruppe von Herrn PD Dr. Horst Zitzelsberger in der Abteilung für molekulare Strahlenbiologie am Helmholtzzentrum für Umwelt und Gesundheit in Neuherberg.

### Promotion

2008 - 2012 Doktorarbeit in der Arbeitsgruppe von Prof. Dr. Olaf Stemmann am Max-Planck-Institut für Biochemie, Martinsried sowie am Lehrstuhl für Genetik der Universität Bayreuth

## 8. PUBLIKATIONSLISTE

Im Rahmen dieser Arbeit entsteht folgende Veröffentlichung:

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Hiermit versichere ich, die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Ferner erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth, den 9.5.2012

Andreas Brown

